BIOCHEMICAL STUDIES ON HAEMOGLOBINS OF TREMATODES AND THEIR HOSTS

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This is to certify that the thesis entitled "Biochemical studies on haemoglobins of trematodes and their hosts" which is being submitted by Mr. Syed Ashfaq Haider, embodies original work done by the candidate himself. The entire work was carried out under my supervision between 1972-1975 and that I allow him to submit the same in fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology of this University.

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ABSTRACT

The occurrence of haemoglobin in trematodes has been reported by many workers and it is, as a matter of fact, quite widespread in Digenea than in any other group of helminths. However, in a very few cases trematode haemoglobins have been compared with their host haemoglobins or with other trematode haemoglobins. The presence of haemoglobin is quite sporadic in the animal kingdom and is interesting from the point of evolution as well as host parasite relationship. Recently it has been detected in several groups of lower animals, but has been seldom reported from parasitic helminths. It is apparently absent in cestodes and there is surprising lack of data on the physical and biochemical nature and source of haemoglobin in trematodes, although a considerable number of these parasites do in fact contain haemoglobin. Studies on haemoglobin of parasites have been confined largely to its spectral characteristics, identification of the pigments, and estimation of the amount present. However, little work has been done in this field of trematode physiology on a comparative basis. In Aligarh, cattle, pigs and fishes harbour various species of trematodes in enormous numbers. These trematodes occur in different habitats and provide a favourable opportunity

to study some of the comparative aspects of the trematode haemoglobins in relation to its host as well as from the point of view of interspecific differences and similarities in trematodes at a molecular level. For this study the author chose the following three host parasite groups: (A). Bubalus bubalis and its trematodes, Gastrothylax crumenifer, Cotylophoron cotylophorum and Gigantocotyle explanatum;(B). Sus scrofa and its trematodes, Fasciolopsis buski, Gastrodiscoides hominis;and (C). Wallago attu and its trematode, Isoparorchis hypselobagri.

The haemoglobins of trematodes and their hosts have been analysed and studied on a comparative basis. Among these, spectral and alkali denaturation studies, estimation of the molecular weight by Sephadex G-100, and electrophoretic studies on polyacrylamide gel were carried out. The absorption spectra of the porphyrins are so characteristics that in many cases they can be used to identify and differentiate various kinds of pigments. In the present study, various derivatives of trematode haemoglobins show characteristic bands and peak maxima and it was established beyond doubt that all pigments are true porphyrins. However, distinct differences were seen in the nature of spectral curves of cyanmet haemochromogens and in all cases, the trematode pigment derivative gives two absorption maxima, in the β and the α region, whereas in the case of host haemoglobin, a characteristic single broad peak in the 538-540 nm region is obtained.

Differences between various haemoglobins can be easily detected by their different susceptibilities to alkali denaturation. The kinetics of alkali denaturation of trematode and hosts haemoglobins were investigated spectrophotometrically at 578 nm, and it was observed that in all cases, the denaturation occurs in a linear fashion of the first order kinetics. It was observed that trematode haemoglobin seems to be more resistant in alkaline solution than does the host haemoglobin, except in the case of Cotylophoron cotylophorum haemoglobin, which was found to be more labile than the cattle haemoglobin. Disc gel electrophoretic studies were carried out on polyacrylamide gel, and it was found out that in all cases the electrophoretic pattern and mobility of trematode haemoglobin was different from that of the host haemoglobin. The host haemoglobins give a slow migrating band in all the three cases under study, while the trematode haemoglobins except Gigantocotyle explanatum and Fasciolopsis buski seem to consist of two fractions. An interesting aspect of the present study is that out of the six trematode parasites, four are amphistomes, and in all cases except Gigantocotyle explanatum, the amphistome haemoglobin appears as two fractions revealing a remarkable parallelism between the electrophoretic pattern of closely related species. This feature of the present study indicates that Cotylophoron cotylophorum, Gastrothylax crumenifer, and Gastrodiscoides hominis are closely related to each other than to Gigantocotyle explanatum. The electrophoretic mobility of Isoparorchis hypselobagri haemoglobin is distinctly different from

the other five trematode haemoglobins as far as electrophoretic mobility is considered, and thus indicating that it is quite distinctly different from the haemoglobins of the other trematode species.

Estimation of the molecular weight of six different species of trematode haemoglobins was carried out on Sephadex G-100. Trematode pigments were chromotographed and their molecular weights were determined on a previously calibrated Sephadex G-100 gel column by first eluting various marker proteins with known molecular weights ranging from 12,000 to 68,000. The molecular weight of trematode pigments range from 17,000 to 21,200 compared with 68,000 in case of the host haemoglobin. The trematode haemoglobins are true porphyrin compounds in the monomeric form occurring in the lowest bilatria. They are obviously proteins of endogenous origin and reflect on the manner of evolution of the tetrameric haemoglobin molecule of higher vertebrates and may be termed as trematode myoglobins.

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INTRODUCTION AND HISTORICAL REVIEW

The term haemoglobin was coined over one hundred years ago, when Hoppe-Seyler (1864) first used it in referring to the pigments of the blood. Today, we understand this term to include the respiratory (oxygen-carrying) proteins of vertebrates. The word "erythrocruorin" (red pigment) was introduced by Lankester (1868) as a general name for all protohaem oxygen-carrying respiratory pigments, without any implication as to the structure or function of the pigment in contrast to the analogous pigments of certain marine worms which he called "chlorocruorins". Smith (1961) is of the view that the term erythrocruorin be used for all known or presumed protohaem respiratory pigments which are known or presumed to be capable of undergoing reversible oxygenation. All erythrocruorins which are known or presumed to function in oxygen storage can be classed as myoglobins.

Studies in recent years have shown that haemoglobin and myoglobin have very similar structures, and that this is probably also true for erythrocruorins (myoglobins). These facts suggest

that there is a phylogenetic relationship between haemoglobin and myoglobin, and the functions of these molecules are transport and storage of oxygen respectively. It also appears probable that these molecules have evolved from a common precursor. Studies on the haemoglobins started at the beginning of the third century. Particularly important were the works of Kuster (1912) and Willstatter (1913) on the porphyrins and the systematic investigations by other workers, which culminated in the complete synthesis of protohaems (Fisher & Zeile, 1929).

As early as 1866, Korber noticed differences in the patterns of denaturation of the various haemoglobins by strong acids or alkali. This species specificity has been confirmed by modern physicochemical methods and also by immunological techniques. The studies of the chemical derivatives of haeme (Haurowitz, 1928) strongly suggested that the prosthetic groups of all haemoglobins were identical. Poldetmann (1932) arrived at a similar conclusion on the basis of his spectroscopic studies. Shortly thereafter Roche and coworkers (1934) provided conclusive proof for this assumption by demonstrating the difference in the amino acid content of various animal haemoglobins. The first end group analysis on haemoglobin was performed by Porter and Sanger (1948). They showed that in adult haemoglobin and horse haemoglobin, the terminal amino group was contributed by valine. In beef, sheep and goat haemoglobins, the terminal

amino groups were valine and methionine. These qualitative results have been confirmed by other workers. In 1938, the Cambridge group of crystallographers began their work on haemoglobin with the purpose of using X-rays to provide information about the external and internal structure of the protein molecule. After many setbacks, a fundamental breakthrough was achieved in 1954, which led towards an understanding of the protein structure (Green et al., 1954). Four years later, a molecular model of myoglobin had been derived (Kendrew et al., 1958) and two years after that, a similar model was available for horse haemoglobin. Thus, the X-ray data provided a picture of the structure of haemoglobin which was entirely consistent with the information obtained by chemical and physico-chemical techniques.

Structure of haemoglobin:

The prosthetic group of haeme proteins is an iron complex of protoporphyrin IX (Fisher & Orth, 1937). Prophyrins are very common in nature and play a fundamental role in biological processes such as photosynthesis, electron transfer and reversible oxygen binding, all apparently quite different functions from one another. Their function depends on the specific properties of their metal complexes (Fe, Cu, Mg) which are coordination compounds of great stability (Martell & Calvin, 1963).

The porphyrins are made up of four pyrrol rings linked together by methane groups, the positions at the corners are occupied by different residues which constitute the side chains. The structure owes many of its properties to the resonating system of bonds between the nitrogen and carbon atoms of the pyrrol rings. The metal occupies a central position in the ring. The coordination linkage of the iron in haeme proteins may be ionic, covalent, or of intermediate character.

In the case of iron porphyrin compounds, hexa coordination of the iron atom is assumed and four of the bonds lie in the plane of the prophyrin ring and are directed towards the pyrrol nitrogen. The other two bonds are available for attachment of other groups and have directions perpendicular to the plane of the ring.

The coordination chemistry of the haeme compounds, their electronic structure, the properties of the metal in these compounds and field theory have been the subject of extensive reviews (Williams, 1956; Griffith & Orgel, 1957; George et al., 1961).

The occurrence and distribution of haemoglobin:

The occurrence and distribution of haemoglobin is quite widespread in the animal kingdom. Haemoglobins have also been

found in leguminous root nodules, in yeasts and in moulds.

Among microorganisms, spectroscopically identifiable haemoglobins are found in ascomycete fungi Neurospora crassa and Penicillium notatum (Keilin & Tissieres, 1953), and in the protozoans Paramecium caudatum and Tetrahymena pyriformis (Keilin & Rylet, 1953).

Leguminous root nodules contain a haemoglobin like pigment with similar properties to those of animal haemoglobin (Sternberg & Virtanen, 1962). Bufolk (1959) found that "leghaemoglobin" could be resolved chromotographically into two major, and into two minor components; the major components possessed molecular weights of 17,500 and 19,500 respectively.

Fox & Veveris (1960) have reviewed the distribution of respiratory pigments, including haemoglobin, among invertebrate animals. Haemoglobins are found among many parasitic nematodes and usually appear to be distinct from the haemoglobins of their respective hosts.

Haemoglobins are also found in many annelids and their physiological and chemical properties have been studied in some detail (Manwell, 1959; Needham, 1969).

Dissolved haemoglobin is found in the blood of some members of the entomostrace crustacea (Fox, 1957). Among the

insects, haemoglobin is somewhat rare in occurrence, being found only in few Diptera and Hemiptera. Haemoglobins are also found in blood of some gastropod molluscs and some echinoderms, and limited studies have been made of their properties, for example, by Yagi *et al.*, (1965) on the molecular weight, absorption spectra and amino acid composition of *Andara inflata*.

Most invertebrate haemoglobins are extracellular and have relatively high molecular weights and low iso-electric points compared with the intracellular haemoglobins of vertebrates. However, haemoglobins of relatively low molecular weights are found in Chironomus, some annelid, species, some nemerteans, and in the lamellibranch mollusc *Arca*. In both, *Arca* and the polychaete *Notomastus*, the haemoglobin, with a molecular weight of about 30,000, is contained within the corpuscles. Such low molecular weight haemoglobins should be contrasted with the haemoglobins of such invertebrates as *Daphnia* (mol. wt. 360,000) and *Planorbis corneus* (mol. wt. 1,300,000) Lyman (1948).

The enormous diversity in the physical characteristics of invertebrate haemoglobins, and their disparate distribution throughout the invertebrate phyla suggest that, they may have evolved independently several times during the course of evolution. At the same time it is necessary to stress that: (a) many

of these large molecular weight invertebrate haemoglobins are in many cases, aggregations of many chain globins, each bound to a haeme group and that (b) absence of haemoglobin cannot be taken to imply absence of globin. Haemoglobins are found throughout the vertebrate phylum and appear to constitute the only respiratory protein of the blood. In most vertebrates, the haemoglobin is tetrameric, each molecule consisting of four globin chains, and each chain being associated with an iron containing porphyrin haem group (Braunitzer, 1958; Muller, 1961a,b). The molecular weight of such vertebrate haemoglobins varies between 61,000 and 72,000, but despite considerable difference in the primary structures of their globin chains, in higher vertebrates the isoelectric points of the proteins are restricted to a pH range of 6.9 - 7.3 and in lower vertebrates to the approximate pH range of 6.0 - 8.0 (Gratzer & Allison, 1960).

Primitive haemoglobins still consists of only a single peptide chain. The haemoglobin of Lampetra has a molecular weight of 17,000 and possesses only one haem group, thus implying it to be monomeric. Svedberg (1933) found that haemoglobins of molecular weights of 17,000 and 34,000 were found in Myxine glutinosa suggesting the presence of both dimers and monomers.

Haemoglobin in Trematodes

Our present knowledge on the respiratory pigment in trematodes appears fragmentary and incomplete, although, according to Lutz and Siddiqi (1967), the occurrence of haemoglobin is much more widespread in Digenea than it has been so far reported. Studies on the haemoglobins of parasites have been confined largely to its spectral characteristics and estimation of the amount present.

Occurrence of haemoglobins have been reported in trematodes like Allassostoma and Telorchis (Wharton, 1941) Dicrocoelium dendriticum and Fasciola hepatica (Van Grembergen, 1949), Fasciola gigantica, Cotylophoron indicum and Gastrothylax crumenifer (Goil, 1959, 1961). Freeman (1963) has reported the occurrence of haemoglobin in the trematode Proctoeces subtenuis, from a stage which is parasitic in a bivalve, which has no haemoglobin. Todd & Ross (1966) using alkaline hematin tests and using a band spectroscope failed to find haemoglobin in F. hepatica without visible caecal contents, though they reported that these flukes have the same iron content as host liver. Van Grembergen (1949) on the other hand found the tissue haemoglobin bands in such animals much clearer than in worms with filled ceca. Lee & Smith (1965) reviewed the subject exhaustively and felt that there was lack of information on the haemo-

globin of trematodes. Recently trematode haemoglobin was studied by Halton (1967) and Lutz & Siddiqi (1967). The latter workers demonstrated spectrophotometrically and electrophoretically that the haemoglobin of F. gigantica is a true porphyrin pigment, distinct from that of the host. These authors for the first time suggested that the parasite haemoglobin is endogenous. More recently, Cain (1969a,b,c) employing gel filtration, ion exchange chromotography and preparative disc electrophoresis determined the molecular weight of F. hepatica haemoglobin which was found to be 17,500 and concluded that the protein resembles vertebrate myoglobin lacking subunits having one haem and containing similar number of amino acids, but half cysteine and taurine were detected in preparations of the parasite haemoglobin.

Besides the work referred to above, little information is available on either the physico-chemical nature of trematode haemoglobins, or on its physiological functions, and there are many gaps and lacunae in our knowledge of this sadly neglected but important field of invertebrate biochemistry and physiology.

STATEMENT OF PROBLEM

It can be seen from the foregoing review of literature, that the present status of our knowledge on the occurrence, distribution and nature of trematode haemoglobin is still very much on the threshold and far from being satisfactory. Much more is desired to be known about the physico-chemical characters and the intriguing function of these coloured proteins. Most of the studies on trematode haemoglobins have been confined largely to its spectral characteristics and only in one or two cases, to electrophoretic analysis. Studies on trematode haemoglobins are important, not only, because they would provide an opportunity to speculate on the biological significance of these pigments in an anaerobic group of organisms, but they would also provide an insight into the intrinsic physiological relationship between host and parasites. Not only such studies will be useful and relevant to the current theories of evolution but at the same time they would also be of considerable interest to trematode taxonomists.

Investigations on haemoglobins of trematodes are obviously important for a better understanding of the invertebrate compara-

tive physiology and biochemistry because functions like O_2 storage and transport, intracellular oxidation and electron transfer are dependent on this class of compounds. Although the significance and function of vertebrate myoglobins is well understood, there has always been some amount of doubt to accept the premise that trematode haemoglobin (or is it myoglobin?) could have similar functions. This appears to be derived from two notions:

1. That parasite haemoglobins are haemoglobin residues of host origin, probably obtained by feeding on host blood.

2. That parasites dwell in oxygen poor habitats and could not make any use of haemoglobin.

Both these contentions have to be examined in the light of recent researches. It seems quite reasonable to assume that if haemoglobins are present in the tissues of the trematodes, there is every possibility that they function in the same manner as do the haemoglobin and myoglobin of the vertebrates. There is surprising lack of data on the haemoglobins in a very large number of trematodes which are important, firstly because they constitute a very large group of the lowest bilatria and secondly as parasites of man and his domestic animals, they cause immense economic losses.

Before detailed studies are carried out on the respiratory function of trematode haemoglobin, it becomes imperative to study the physico-chemical properties of as many different and closely allied species of trematodes as possible. Such a study would not only reveal the true nature of trematode haemoglobin, but will also provide evidences if any on the phylogenetic relationship of the trematodes on a molecular level. According to Florkin (1963)".....proteins such as haemoglobins change in structure along the phylogenetic derivation of the organism containing them and that it is possible to follow the changes for example from annelid haemoglobin to chlorocruorins of chlorohemians along a well defined segment of phylogeny and to recognize an aspect of the descent with change at the molecular level, as well as a common origin for different proteins. On the other hand, the existence, of haemoglobins, haemocyanins and haemery-thrins was an example of analogy. In this way common iiioii descent and not common biological action of the molecule appeared to me as a leading concept into which a comparative biochemistry could be built".

In Aligarh, cattle, pigs, and fishes harbour, in enormous numbers, various species of trematodes. These trematodes occur in different habitats and provide a favourable opportunity to study some of the comparative aspects of the trematode haemoglobins in relation to its host as well as from the point of view of interspecific differences and similarities in trematodes at a molecular level.

For this study, the author chose the three host-parasite groups:

Host	Site	Trematode parasites
Bubalus bubalis (water buffalo)	Rumen	(1) Cotylophoron cotylophorum
	Rumen	(2) Gastrothylax crumenifer
	Liver	(3) Fasciolopsis buski
Sus scrofa (Pig)	Intestine	(1) Fasciolopsis buski
	Caecum	(2) Gastrodiscoides hominis
Wallago attu (Catfish)	Urinary bladder	Isoparorchis hypselobagri

The haemoglobins of trematodes and their hosts have been analysed and studied comparatively. Among these, spectral and electrophoretic studies, rate of alkali denaturation and molecular weight determination have been carried out, using modern and reliable techniques. It is hoped that whatever little has been accomplished by the present investigator would stimulate further research work on this topic for a comparatively less studied field of trematode biochemistry.

MATERIALS AND METHODS

The cattle trematodes, *Gastrothylax crumenifer* and *Cotylophoron cotylophorum* from the rumen, and *Gigantocotyle explanatum* from the bile ducts were collected from the local abattoir, soon after the animals were slaughtered; *Fasciolopsis buski* from the small intestine and *Gastrodiscoides hominis* from the caecum or large intestine were recovered from pigs slaughtered at the local U.P. Government Central Dairy Farm. *Isoparorchis hypselobagri* were collected from the swim bladder of the catfish, *Wallago attu*, obtained from the local fish market. The worms were transported to the laboratory in vacuum flasks containing appropriate salines. Tyrode saline was used for mammalian trematodes, and modified Ringer's (Forster & Taggart, 1960) containing NaCl 100mM, KCl 2.5mM, $CaCl_2$ 1.5mM, $MgCl_2$ 1.0mM, NaH_2PO_4 0.5mM, and $NaHCO_3$ 5mM was used for fish trematode.

In the laboratory, the cattle and pig trematodes were washed twice with Tyrode saline and stored in 2-3 ml of Tris-HCl buffer (pH 7.4) in a deep freeze. The fish trematodes were

similarly washed in fish saline and stored in 2-3 ml of Tris-HCl buffer. The haemoglobin was extracted by alternately freezing and thawing the worms several times after the method described by Wharton (1961). The extract was centrifuged at 6000 rpm to remove the coarse debris and the clear supernatant fluid was pipetted out for subsequent studies. This method of extraction of trematode haemoglobins proved to be superior over homogenisation of the worms especially when facilities for ultracentrifugation were not always available.

The host blood was collected in 10% EDTA or ACD (Acid citrate dextrose) which was used as an anticoagulant. The blood was spun in a centrifuge to pack the red cells, after which the supernatant plasma was removed. These cells were washed three times with isotonic saline and then lysed in an equal volume of deionized water. After hemolysis, cell stroma was separated from the solution by centrifuging the hemolysate at 6000 rpm for 10 minutes. The pure haemoglobin solution was then filtered through one layer of Whatmann filter paper No.42, or cellulose acetate thimbles.

Spectrophotometric Analysis:

Five different derivatives of both trematode and their host haemoglobins were prepared as follows :

a. Oxyhaemoglobin:

For the study of oxyhaemoglobin, three millilitres of

freshly extracted haemoglobin was used.

b. Reduced Haemoglobin:

Reduced haemoglobin was prepared by adding a slight excess of about 2 mg of sodium dithionite crystals to 3 ml of haemoglobin solution, and the cuvette was immediately stoppered to exclude any atmospheric oxygen.

c. Carbonmonoxyhaemoglobin:

Gaseous carbon monoxide was bubbled through 3 ml of the haemoglobin solution for 15 minutes to obtain carbonmonoxyhaemoglobin. Carbon monoxide was manufactured by the dehydration of formic acid by concentrated sulphuric acid. Formic acid was added drop-wise to the sulphuric acid in a system with only one outlet.

d. Cyanmethaemoglobin:

The cyanmethaemoglobin was prepared by adding 0.05 ml of undiluted oxyhaemoglobin to 3 ml of freshly prepared Drabkin's solution (1g $NaCO_3$, 50mg of KCN and 200mg of $K_3Fe(CN)_6$ in 1 litre of distilled water). The mixture was shaken.

e. Pyridine haemoglobin:

Pyridine haemochrome was prepared by adding 0.8 ml of pyridine and 0.2 ml of 4N sodium hydroxide to 3 ml of haemoglobin solution.

Absorption maxima of the above mentioned derivatives were determined at 380 to 700 nm, on a 'Beckman DU2' spectrophotometer using a cuvette of 1 cm light path.

Protein Estimations:

Protein estimation of the various samples of trematode haemoglobins was made by the method of Lowry et al., (1951), using a 'Spectronic 20' spectrophotometer, and the protein concentration was read on a previously established standard curve.

Alkali Denaturation:

The progress of alkali denaturation of oxyhaemoglobins of trematodes and their hosts was automatically recorded on a Beckman DU 2A ratio recording spectrophotometer at 575 nm. The reaction mixture contained 2.5 ml of oxyhaemoglobin and 0.5 ml of 0.5 M NaOH so that the final concentration of the alkali achieved was 0.1 M and the pH of 13.4. The percent denaturation of the oxyhaemoglobins was calculated per minute as a function of change in absorbance. The degree of denaturation has been expressed as log , undenatured oxyhaemoglobin against time.

Disc Electrophoresis:

(1) Acrylamide, (2) N,N'Methylenobisacrylamide, (3) Riboflavin, (4) N,N,N',N'-Tetramethyl ethylene diamine were obtained from Koch-Light Laboratories, England. Ammonium persulphate and Glycine from Riedal, Germany, Tris (hydroxymethyl) aminomethane, and EDTA from BDH (England).

For electrophoresis, purified and concentrated carbonmonoxy derivatives of haemoglobin samples from trematodes and their hosts were studied for their electrophoretic mobilities, by the acrylamide gel electrophoresis method (Davis, 1964). The tank assembly for disc electrophoresis apparatus was fabricated in the laboratory (Fig. 1). An A.C. mains power pack fashioned with D.C. current output was used as the power supply.

Prior to electrophoresis, the gel tubes (7 cm long with 0.6 mm inner diameter, were first washed with a detergent (Teepol, Burmah Shell) and then with chromic acid. The tubes were finally rinsed with deionized water and were siliconized either with 1% solution of Silicleat (Clay Adam, N.Y.. or with 7% solution of dichlorodimethyl silane in chloroform.

Disc electrophoresis was performed in columns of polyacrylamide gel consisting of 3 sections: (i) a large pore anticonvection gel containing the protein sample, (ii) a large pore spacer gel, and (iii) a small pore gel, in which electrophoretic separation was accomplished.

The various reagents were prepared as follows:

Stock Solutions:

Solution A

1N HCl	48.00 ml
Tris	36.60 g
Temed	0.23 ml
Distilled water	100.00 ml (pH 8.9)

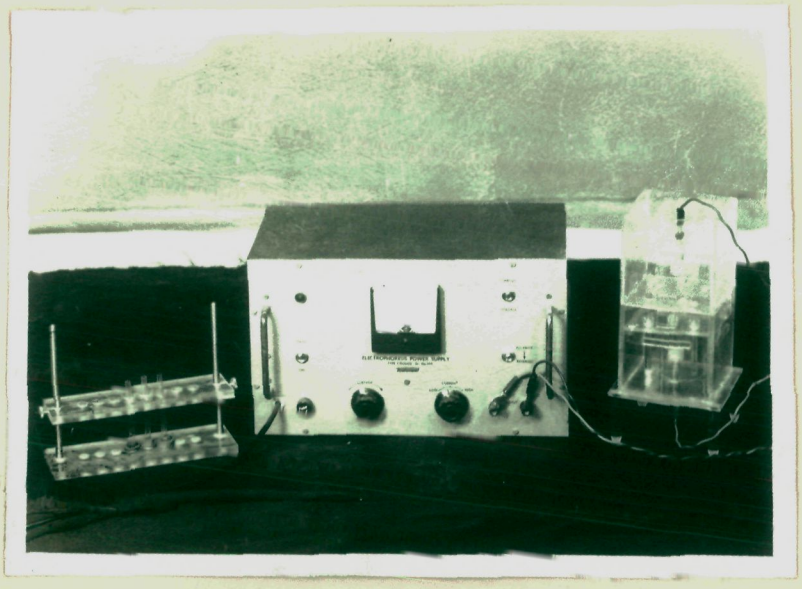


Fig. 1. Disc electrophoresis apparatus

Solution B

IN HCl	48.00 ml
Tris	5.98 g
Temed	0.46 ml
Distilled water	100.00 ml

pH 6.7 was adjusted by titrating with IN HCl

Solution C

Acrylamide	28.00 g
BIS	0.7350 g
Distilled water	100.00 ml

Solution D

Acrylamide	10.00 g
BIS	2.5 g
Distilled water	100.00 ml

Solution E

Riboflavin	4.00 mg
Distilled water	100.00 ml

Solution F

Sucrose	40.00 g
Distilled water	100.00 ml

Small pore solution

1 part A
2 parts C
1 part water
(pH 8.8-9.0)

This was mixed with an equal quantity of 0.14% of ammonium persulphate solution.

Large pore solution

1 part B
2 parts D
1 part E
4 parts F

pH = (6.6-6.8)

Stock Buffer Solution for Reservoirs

Tris	6.0	g
Glycine	28.8	g
Distilled water	1	litre

(pH 8.3)

(1/10th strength of this buffer solution was used).

Each tube was filled with a 0.85 ml of small pore gel solution. This solution was clearly overlaid with 0.1 ml of distilled water, and was allowed to stand for 30 minutes. The water layer was removed and 0.15 ml of large pore gel solution was added and carefully overlaid with 0.1 ml of distilled water. A day light fluorescent lamp was placed over the tubes for the polymerisation of the large pore gel which was accomplished within 15 minutes. The water layer was removed, and the large pore gel solution (0.15 ml) which was mixed with 40-50 ml of the protein sample was added. The sample layer was again polymerised for 15 minutes in the manner described above. The remaining space in each gel tube was then filled with buffer solution.

An alternative and relatively simple method of sample application was also used, in which the use of sample with

spacer gels was omitted, the sample which consisted of 40 to 80 ul of protein, 3 ul of bromophenol blue (0.05%) and one drop of glycerol were mixed and applied to each gel under a layer of buffer just above the small pore solution. The latter method of sample application gave better resolution and moreover the period of time of each electrophoretic run was almost reduced to half.

Electrophoresis was carried out at room temperature by using 7% acrylamide gels, standard Tris-glycine buffer (pH 8.3) and constant current of 3 mA/tube till the tracking bromophenol blue dye reached near the bottom of the tube. This usually did not take more than one hour. When electrophoresis was completed the gels were immediately removed from the glass tubes by gently rimming them with a 22 gauge needle through which a thin stream of water was made to pass. Before staining the major haemoglobin *xi.* fractions appeared as red bands.

These bands were selectively stained by the method of Ornstein (1965) using benzidine reagent, which was prepared as follows: A solution of 16 g sodium acetate in 100 ml of 7% acetic acid was saturated with EDTA. After filtration, this solution was saturated with benzidine and refiltered. Immediately 33r before use 0.1 - 0.2 ml of 3% hydrogen peroxide was added to 10 ml of benzidine reagent. The haemoglobin bands stained green or greenish blue, and turned dark green in about 24 hours.

During the course of these experiments it was experienced that the hemoglobin bands appear completely in about 24 hours, by which time the bromophenol blue tracking dye band disappears, since it is soluble in the staining solution. The same, therefore could not be photographed along with the haemoglobin bands. This difficulty was overcome by inserting a small piece of black nylon bristle at the middle of the bromophenol blue band. This method proved very successful. The gels were photographed against diffuse light.

Partial purification of haemoglobin and determination of molecular weight:

Partial purification of the parasite haemoglobins was carried out by ammonium sulphate precipitation method.

The haemoglobin sample was centrifuged at 30,000 rpm on Beckman preparatory untracentrifuge at 4°C for about one hour. The supernatant liquid was dialysed overnight against Tris-HCl (pH 7.4) at 4°C and again centrifuged at 30,000 rpm at 4°C. Solid ammonium sulphate (Analar) was added to bring the solution to an initial saturation of 50%, and then more ammonium sulphate was added to bring the solution to a final saturation of 75%. The solution was dialysed overnight at 4°C and retained for determination of molecular weight. Dialysis was carried out in a dialysing bag (No.44 66-A, Arthur H. Thomas). In this way two fold purification of the haemoglobin was obtained.

Horse heart cytochrome and sperm whale myoglobin were obtained from Koch-Light Laboratories. Egg albumin, and bovine serum albumin were obtained from Sigma; papain and bovine haemoglobin were obtained from Biochemicals Unit, Patel Chest Institute, New Delhi. Sephadex G-100 (medium) was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

The sephadex gel was suspended in enough buffer and properly stirred to remove any air bubbles. It was then allowed to swell for 3 days at room temperature. A glass column (1.7 x 40cm) was used for gel filtration and a plug of glass wool and few glass beads were placed at the bottom of the column, which was then approximately half filled with 0.6M Tris-HCl buffer (pH 7.4), and the gel suspension was carefully added to bring the level to the top of the column. After a 5 to 6 cm of layer of sephadex had settled to the bottom, the column outlet was gently opened and the settling of the gel continued. More gel suspended in buffer was added to the column until a bed height of 40-41 cms was obtained, and then a buffer reservoir was connected to the top of the column and the flow of the column was maintained at a rate of 16 ml/hour for 2 days. By this time the gel bed had settled to a constant height, which was then adjusted to 40 cm (bed Vol. 90 ml). Even packing of the column was checked by watching the passage through it of a band of blue dextran.

Application of the sample:

The protein was added to the top of the gel bed in 1 ml of the buffer. Under flow, the protein was washed into the gel with additional buffer, and the buffer was then added above the gel. The column was then calibrated by determining the elution volume of several marker proteins of different but known molecular weights, and a fraction of 2.5 ml each was collected at a flow rate of 12 ml/hour. The absorbance of each fraction was measured at 280 nm on a Beckman DU2 spectrophotometer. Following marker proteins of different known molecular weights - cytochrome c (12,000); sperm whale myoglobin (17,000); egg albumin (43,000); papain (21,000), bovine serum albumin 68,000), and bovine haemoglobin (67,000) were used. The elution volume of partially purified haemoglobin of different species of trematodes was then determined and the partition coefficient for each protein was calculated from the formula proposed by Andrew (1964):

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

where, V_e is the elution volume, V_o the void volume (elution volume of blue dextran), and V_t the total volume of the gel bed.

The molecular weights of the known marker proteins were plotted against their Kav values and a straight line curve was

obtained. The molecular weights of trematode haemoglobins were determined on the abscissa by extrapolating the K_{RV} values obtained experimentally on the ordinate.

RESULTS

SPECTRO-PHOTOMETRIC ANALYSIS:

The spectrophotometric analysis of five derivatives of haemoglobins of different trematodes and their hosts were made. The derivatives studied were oxyhaemoglobin, carbonmonoxyhaemoglobin, reduced haemoglobin, cyanmethaemoglobin and pyridine haemochrome. In all cases, haemoglobins of similar concentrations were used for spectral studies. The absorption maxima are summarized in Table I - III and the spectral curves are shown in Figs. 2-6. For each sample, several runs were made, and the absorption peaks obtained for each species were consistent. The reproducibility of the recorded peaks fell within the limits of experimental error; imprecision resulting from peak shifts of 1-2 mm were therefore ignored. For the sake of avoiding confusion, each host parasite system has been dealt with separately under each derivative.

(1) Oxyhaemoglobin:

(a) Cattle trematodes:

For oxyhaemoglobin, spectral studies were made on freshly extracted haemoglobin. It can be seen that not only the

Fig. 2. Spectrophotometric comparision of trematode and host oxyhaemoglobins in clarified homogenates. Samples were adjusted to have the same absorbance at 540 nm. The soret bands have been reduced by a factor of 6.

A. Notylophoron notylophorum

B. Gastrothylax crumenifer

C. Gigantocotyle explanatum

D. Bubalus bubalis (Cattle)

E. Fasciolopsis buski

F. Gastrodiscoides hominis

G. Sus scrofa (Pig)

H. Isoparorchis hypselobagri

I. Wallago attu (Catfish)

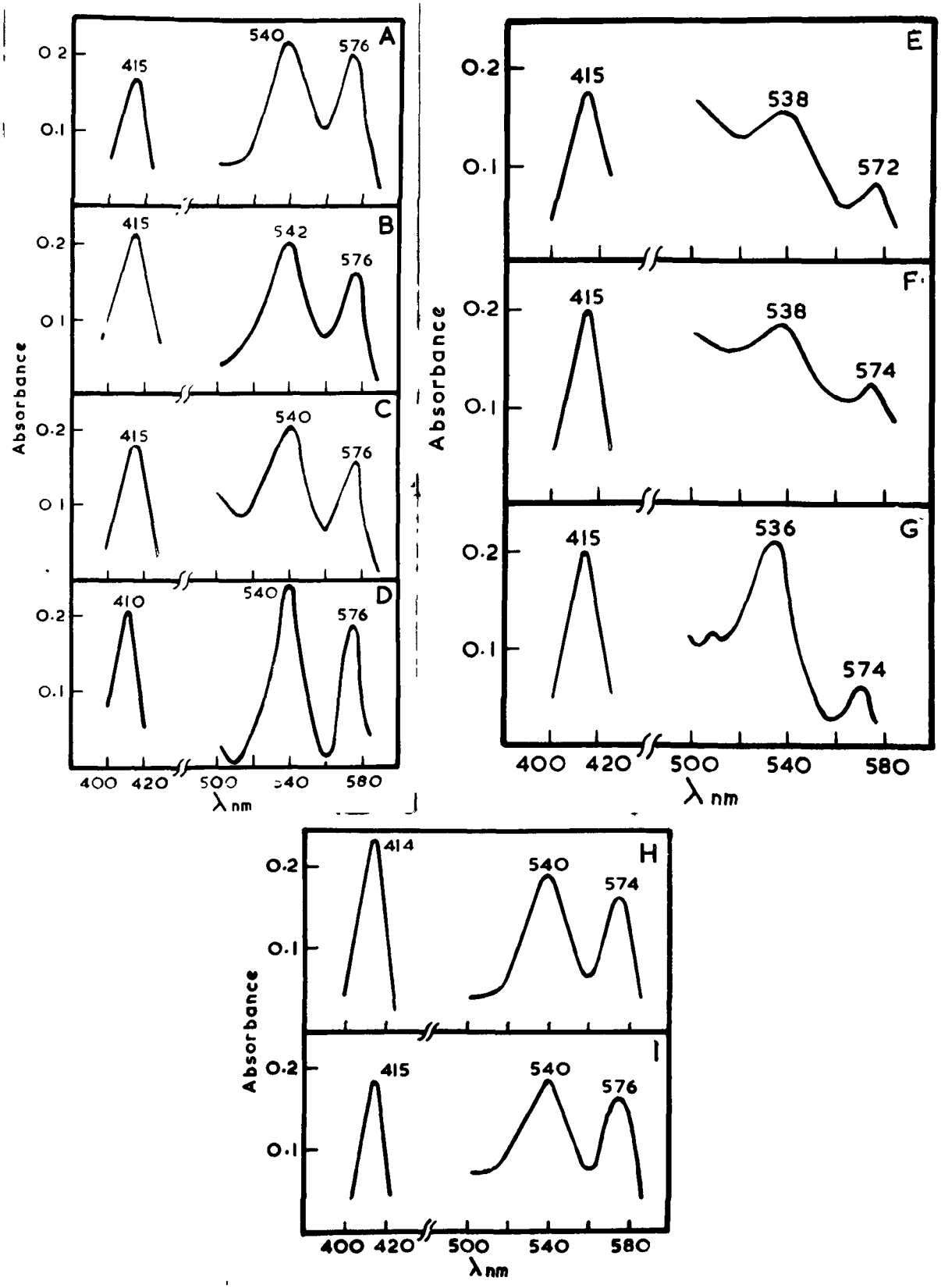

A
415
540
576
B
415
542
576
C
415
540
576
D
410
540
576
E
415
538
572
F
415
538
574
G
415
536
574
H
414
540
574
I
415
540
576
Absorbance
0.2
0.1
400 420 500 540 580
λ nm

FIGURE 2

absorption maxima of cattle trematodes and buffalo haemoglobin occur very near to each other, but the nature of the spectral curves is also very much identical. Further, the β peak is slightly higher than the α peak in all cases.

(b) Pig trematodes:

The nature of absorption curves of the pig trematodes is different from the curves of other trematodes under study, however, the absorption maxima of pig trematodes and host haemoglobins are very close to each other, and moreover, the β peak is comparatively higher than the α peak as compared to other host-parasite haemoglobins.

(c) Fish trematodes:

Same is the case with Isoparorchis hypselobagri and Wallago attu oxyhaemoglobins. In the present case also, the absorption maxima of both the host and parasite haemoglobins are almost identical, with the β peak being slightly higher than the α peak.

(2) Carbonmonoxyhaemoglobin:

For carbonmonoxyhaemoglobin, the nature of the absorption curve is very much similar to the absorption curve of oxyhaemoglobin, but the absorption maxima are at shorter wave lengths than oxyhaemoglobin, in addition, the soret band characteristically

Fig. 3. Spectrophotometric comparision of trematode and host carbonmonoxyhaemoglobins in clarified homogenates. Samples were adjusted to have the same absorbance at 540 nm. The soret bands have been reduced by a factor of 5.

A. Cotylophoron cotylophorum

B. Gastrothylax crumenifer

C. Gigantocotyle explanatum

D. Bubalus bubalis (Cattle)

E. Fasciolopsis buski

F. Gastrodiscoides hominis

G. Sus scrofa (Pig)

H. Isoparorchis hypselobagri

I. Wallago attu (Catfish)

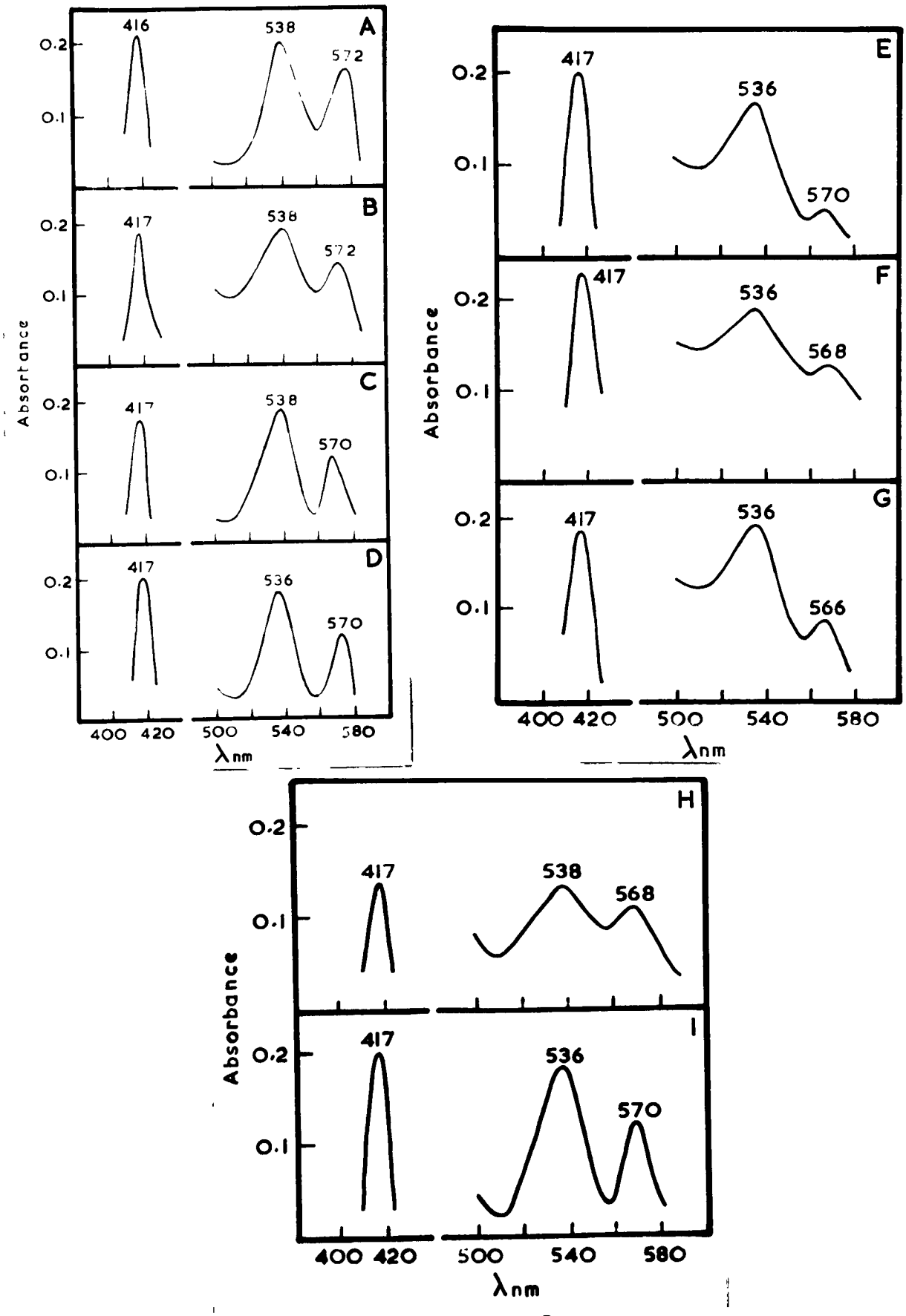

A
416
538
572
B
417
538
572
C
417
538
570
D
417
536
570
E
417
536
570
F
417
536
568
G
417
536
566
H
417
538
568
I
417
536
570
0.2
0.1
400 420
500 540 580
λnm
Absorbance

FIGURE 3

shifts to 417 nm. In all cases the β peak is slightly higher than the α peak.

(3) Reduced haemoglobin:

Deoxygenation of haemoglobin in solution by the addition of sodium dithionite causes the disappearance of the β and the α peaks and a single broad band appears in the yellow green region. In addition, the soret band of reduced haemoglobin is shifted to 427-428 nm.

(a) Cattle trematodes:

The reduced Gastrothylax crumenifer and Cotylophoron cotylophorum haemoglobins give broad bands between 550-64 nm; however, the reduced Gigantocotyle explanatum haemoglobin gives a comparative less broad band at 556-58 nm. Similarly the host haemoglobin gives an absorption peak at 556 nm.

(b) Pig trematodes:

As in the case of cattle trematode haemoglobins the pig trematode haemoglobins also show broad bands between 554-62 nm while the pig haemoglobin shows a lesser broad band between 558-66 nm.

(c) Fish trematode:

The absorption curve for reduced haemoglobin of fish trematode and its host, follow the same pattern as described in

Fig. 4. Spectrophotometric comparision of trematode and host reduced haemoglobins in clarified homogenates. Samples were adjusted to have the same absorbance at 540 nm. The soret bands have been reduced by a factor of 5.

A. Cotylophoron cotylophorum

B. Gastrothylax crumenifer

C. Giganticotyle explanatum

D. Bubalus bubalis (Cattle)

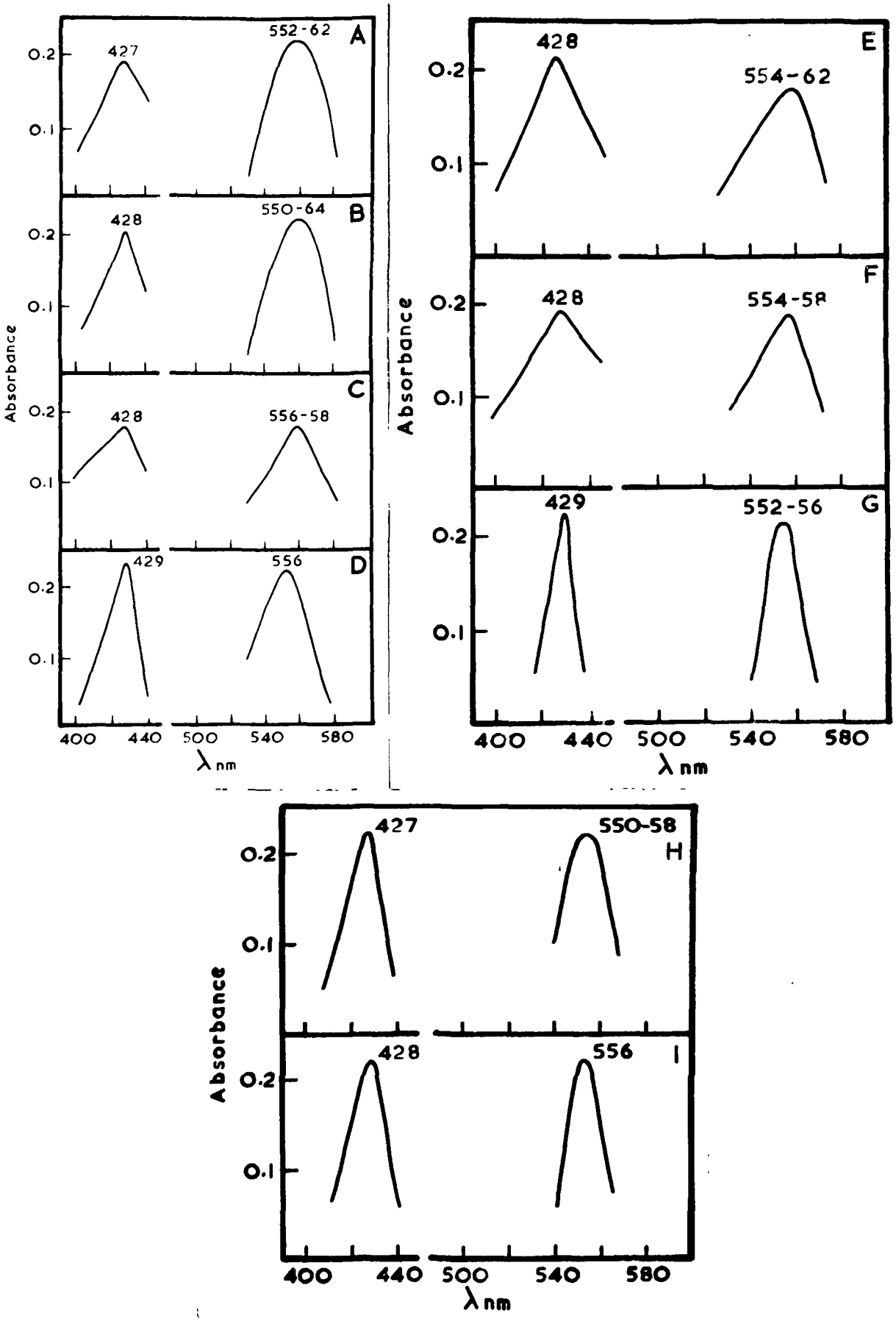
E. Fasciolopsis buski

F. Gastrodiscoides hominis

G. Sus serofa (Pig)

H. Isoparorchis hypselobagri

I. Wallago attu (Catfish)


A
427
552-62
B
428
550-64
C
428
556-58
D
429
556
E
428
554-62
F
428
554-58
G
429
552-56
H
427
550-58
I
428
556
0.2
0.1
Absorbance
400
440
500
540
580
λ nm

the case of cattle and pig trematodes. Reduced Isonarorchis krauslabaari haemoglobin gives a broad band between 550-558 nm, while the host haemoglobin at 556 nm.

(4) Cyanmethaemoglobin:

When Drabkin's solution is added to haemoglobin, cyanmethaemoglobin is formed. The absorption maxima of the soret band of the cyanmethaemoglobin occurs at 417-418 nm.

(a) Cattle trematodes:

It can be seen that the absorption curves of cyanmethaemoglobin of cattle trematodes are altogether different from the curve obtained for buffalo cyanmethaemoglobin. While the trematode haemoglobins show two absorption maxima in addition to the soret band, the host haemoglobin gives a single broad band in the β region i.e., between 534-540 nm. However, in case of the Gigantocotyle explanatum haemoglobin the α peak is reduced considerably.

(b) Pig trematodes:

The nature of the absorption curves of the pig trematode cyanmethaemoglobins are in keeping with the same pattern as seen in other trematode haemoglobins, i.e., both the β and the α peaks are present between 536-540 nm and 570-572 nm region, while the pig cyanmethaemoglobin shows a single broad band between 534-538 nm.

Fig. 5. Spectrophotometric comparision of trematode and host cyanmethaemoglobins in clarified homogenates. Samples were adjusted to have the same absorbance at 540 nm. The soret bands have been reduced by a factor of 5.

A. Cotylophoron cotylophorum

B. Gastrothylax crumenifer

C. Gigantocotyle explanatum

D. Bubalus bubalis (Cattle)

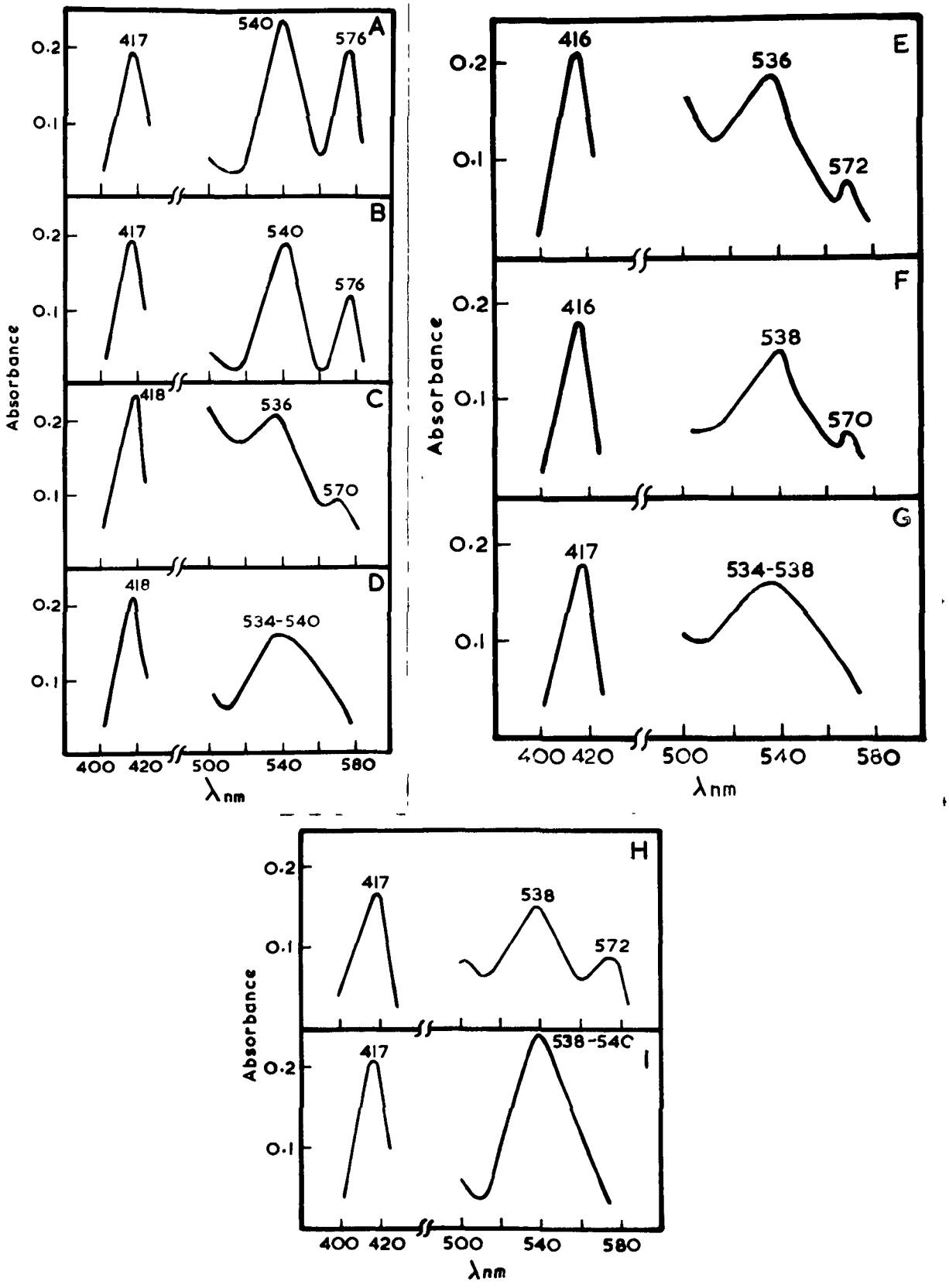
E. Fasciolopsis buski

F. Gastrodiscoides hominis

G. Sus scrofa (Pig)

H. Isoparorchis hypselobagri

I. Wallago attu (Catfish)


A
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B
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C
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536
570
D
418
534-540
E
416
536
572
F
416
538
570
G
417
534-538
H
417
538
572
I
417
538-540
0.2
0.1
Absorbance
400 420 500 540 580
λnm

FIGURE 5

(c) Fish trematodes:

The absorption curve of Isoparorchis hypsalobagri cyanmethaemoglobin also shows two peaks in the β and the α regions, while the host cyanmethaemoglobin gives a single broad band at 538-540 nm.

(5) Pyridine haemoglobin:

The pyridine haemochrome, prepared by the addition of pyridine and sodium hydroxide to a haemoglobin solution, results in characteristic shifting of the soret band from 410 to 394-395 nm.

(a) Cattle trematodes:

The nature of the absorption curve of pyridine haemochrome of cattle haemoglobin is very much different from the absorption curves obtained for the trematode haemoglobin derivatives. The pyridine haemochromes of trematode haemoglobins are characterised by a sharp peak at 554 nm for Cotylophoron cotylophorum; 556 nm for Gastrothylax crumenifer, and 554 for Gigantocotyle explanatum. In case of Cotylophoron cotylophorum and Gigantocotyle explanatum minor shoulders were also observed at 522 and 516 nm respectively. The pyridine haemochrome of buffalo haemoglobin gives a single broad band at 570 nm.

Fig. 6. Spectrophotometric comparision of trematode and host pyridine haemoglobins in clarified homogenates. Samples were adjusted to have the same absorbance at 540 nm. The soret bands have been reduced by a factor of 6.

A. Cotylophoron cotylophorum

B. Gastrothylax crumenifer

C. Gigantocotyle explanatum

D. Bubalus bubalis (Cattle)

E. Fasciolopsis buski

F. Gastrodiscoides hominis

G. Sus scrofa (Pig)

H. Isoparorchis hypselobagri

I. Wallago attu (Catfish)

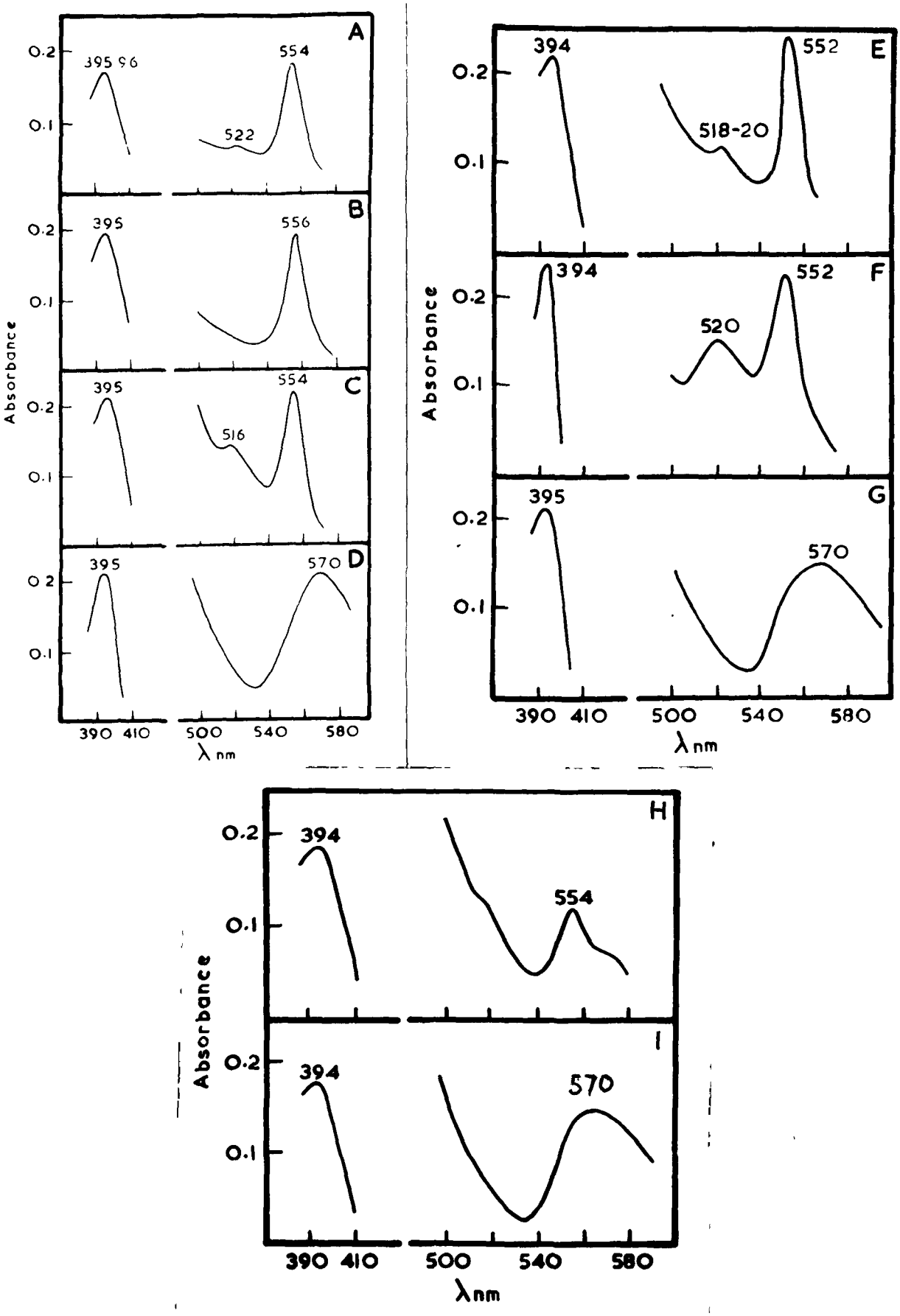
A
B
C
D
E
F
G
H
I
Absorbance
λ nm
395 96
554
522
395
556
554
516
570
394
552
518-20
520
390 410
500 540 580
0.2
0.1

FIGURE 6

(b) Pig trematodes:

The nature of the absorption spectra of pyridine haemochrome of pig trematodes and their host are consistent with the pattern obtained for pyridine haemochromes of cattle trematodes and their host. Fasciolopsis buski gives a sharp peak at 552 nm and a minor hump at 518-520 nm; Gastrodiscoides hominis gives a sharp peak at 522 nm and a major hump at 520 nm. The pyridine haemochrome of pig haemoglobin gives a single broad band at 570 nm.

(c) Fish trematode:

Same is the case with the pyridine haemochrome of Isoparorchis hypselobagri and Wallago attu. Isoparorchis hypselobagri pyridine haemoglobin derivative is characterised by a sharp peak at 554 nm, while that of the host at 570 nm.

TABLE I. ABSORPTION MAXIMA OF HAEMOGLOBIN DERIVATIVES OF CATTLE TREMATODES AND THEIR HOST IN nm (TRIS-HCl BUFFER pH 8.2).

Species	Oxy-Hb			Carboxy-Hb			Deoxy-Hb		Cyanmet-Hb			Pyridine-Hb			Span*
	Soret	β	α	Soret	β	α	Soret	(β-α)	Soret	β	α	Soret		α	
Trematodes:															
[redacted]	415	540	576	416	536	572	427	550-62	417	540	576	395	-	565	4
[redacted]	415	542	576	417	538	572	428	550-64	417	540	576	396	-	566	4
[redacted]	415	540	576	417	538	570	428	556-68	418	536	570	395	516	554	6
Host:															
[redacted]	410	540	576	417	536	570	429	556	-	418(534-540)		395	-	570	6

*(Span in nm between α bands of oxy and carbonmoxide haemoglobins).

TABLE II. ABSORPTION MAXIMA OF HAEMOGLOBIN DERIVATIVES OF PIG TREMATODES AND THEIR HOST IN nm (TRIS-HCl BUFFER pH 8.3).

Species	Oxy-Hb			Carboxy-Hb			Deoxy-Hb		Cyanmet-Hb			Pyridine-Hb			Span*
	Soret	β	α	Soret	β	α	Soret	β-α	Soret	β	α	Soret	β	α	
Trematodes:															
[illegible]	415	536	572	417	536	570	428	564-62	416	536	572	394	518	552	4
[illegible]	415	538	574	417	536	568	428	564-68	416	540	570	396	530	562	6
Host:															
[illegible] (pig)	415	536	574	417	536	566	429	562-66	417	534-538		396	-	570	6

* (Span in nm between α bands of oxy and carbonmoxide haemoglobins).

TABLE III. ABSORPTION MAXIMA OF HAEMOGLOBIN DERIVATIVES OF FISH TREMATODES AND THEIR HOST IN nm (TRIS-HCl BUFFER, pH 8.3).

Species	Oxy-Hb			Carboxy-Hb			Deoxy-Hb		Cyanmet-Hb			Pyridine-Hb		Span*
	Soret	β	α	Soret	β	α	Soret	(β-α)	Soret	β	α	Soret	β	
Trematoda:														
Isoparorchis hypselobagri	414	540	574	417	538	568	427	550-58	417	538	572	394	554	6
Host:														
Wallago attu	415	540	576	417	536	570	428	556	417	538-40		396	538	6

70

* (Span in nm between α bands of oxy and carbonmoxide haemoglobins).

ALKALI DENATURATION:

The results of the alkali denaturation of oxyhaemoglobins of different species of trematodes and their hosts are shown in Figs. 7-9, and the rate and extent of alkali denaturation have been given in Tables IV and V.

Cattle trematodes:

The rate and percent denaturation of haemoglobins of Bubalus bubalis and its parasites, Gastrothylax crumenifer, Cotylophoron cotylophorum and Gigantocotyle explanatum are shown in Fig. 7, and Table IV. It is quite obvious from the data presented that Gigantocotyle explanatum and Gastrothylax crumenifer haemoglobins seem to be more resistant to alkali than the host haemoglobin. However, comparatively speaking Cotylophoron cotylophorum haemoglobin appears to be relatively more alkali labile than the other two trematode haemoglobins. The percent denaturation of trematode haemoglobins of Gigantocotyle explanatum, Gastrothylax crumenifer and Cotylophoron cotylophorum was found to be 25.0, 34.2, 44.3 respectively in 16 minutes while in the case of host haemoglobin it was also 43.0. Therefore, it

can be concluded that the haemoglobin of Gigantocotyle explanatum was slightly more resistant to alkali followed by Gastrothylax crumenifer and Cotylophoron cotylophorum haemoglobins.

Pig trematodes:

The denaturation rate of pig oxyhaemoglobin which was 5.05% per minute was about 1.5 times higher than the rate of alkali denaturation of Gastrodiscoides hominis and Fasciolopsis buski haemoglobins. It is evident from Fig. 8, that the haemoglobin of Fasciolopsis buski was initially resistant to alkali for the first 8 minutes, while the haemoglobin of Gastrodiscoides was alkali labile, though only slightly from the very beginning. The percent denaturation of Gastrodiscoides hominis and Fasciolopsis buski haemoglobins in 16 minutes was found to be 31 and 20 respectively, while that of the pig haemoglobin it was 66.3.

Fish trematodes:

In this host parasite system the haemoglobins of fish and its trematode appear to be quite alkali labile, as it is evident from Fig. 9. The rate of alkali denaturation of fish haemoglobin was found to be 9.42% per minute while in the case of Isoparorchis hypselobagri haemoglobin it was 6.68% per minute. Another difference between the two haemoglobins is that in the case of fish haemoglobin almost complete denaturation occurs in 16 minutes whereas in the case of Isoparorchis hypselobagri haemoglobin it was about 84% in the same period of time.

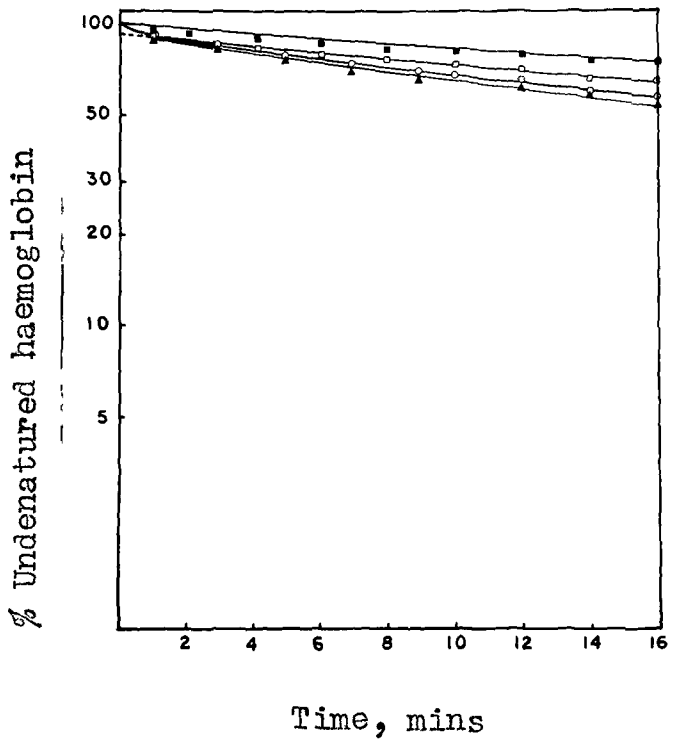

Fig. 7. Alkali denaturation of haemoglobins of cattle and cattle trematodes in 0.5 molar NaOH.

■ Gigantocotyle explanatum

□ Gastrothylax crumenifer

○ Bubalus bubalis (cattle)

▲ Cotylophoron cotylophorum

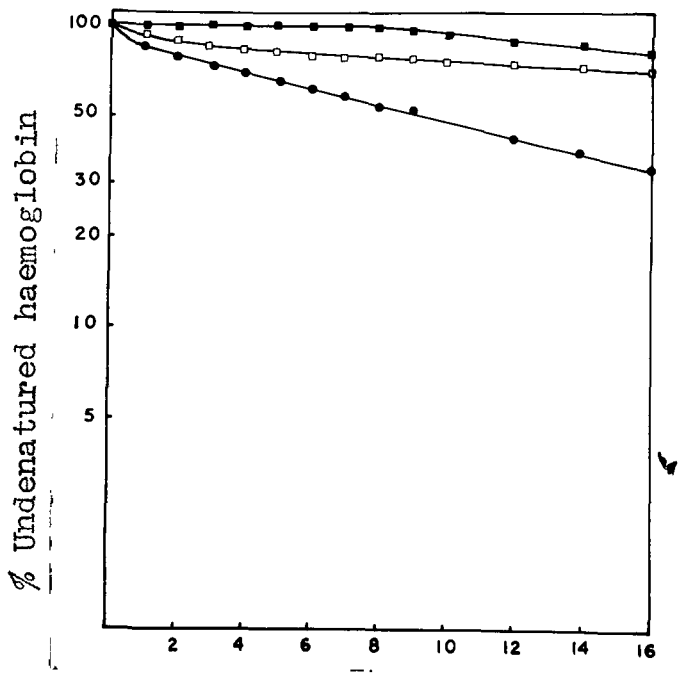

Fig. 8. Alkali denaturation of haemoglobins of pig and pig trematodes in 0.5 molar NaOH.

- ■ Fasciolopsis buski
- □ Gastrodiscoides hominis
- ● Sus scrofa (pig)

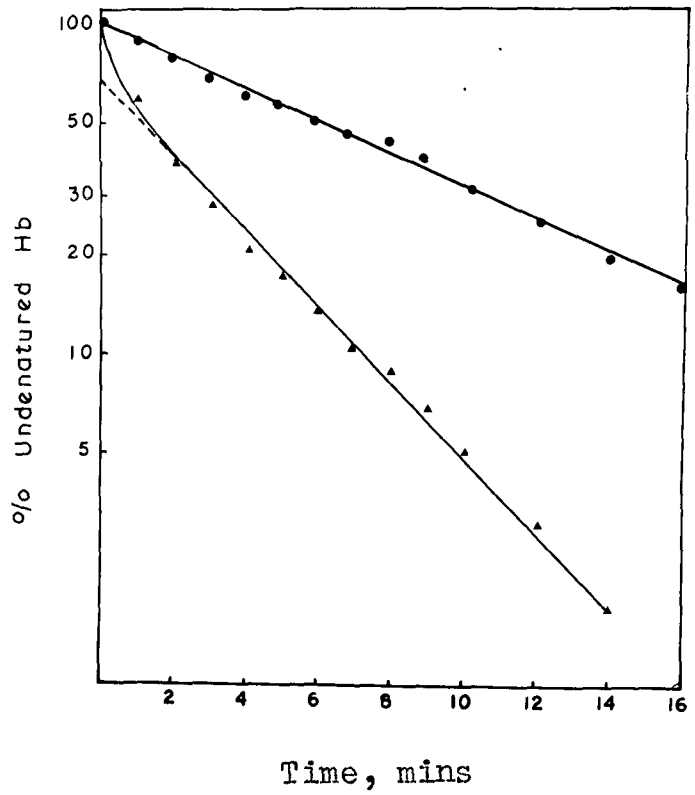

Fig. 9. Alkali denaturation of haemoglobins of fish and fish trematodes in 0.5 molar NaOH.

● Isoparorchis hypselobagri

▲ Wallago attu (Catfish)

TABLE IV. RATE OF ALKALI DENATURATION OF DIFFERENT HAEMOGLOBINS OF TREMATODES AND THEIR HOSTS.

Species	% Denaturation per minute
Trematodes:	
Gastrothylax crumenifer	2.45
Cotylophoron cotylophorum	3.14
Gigantocotyle explanatum	1.88
Host:	
Bubalus bubalis	2.76
Trematodes:	
Fasciolopsis buski	3.30
Gastrodiscoides hominis	2.30
Host:	
Sus scrofa	5.05
Trematode:	
Isoparorchis hypselobagri	6.60
Host:	
Wallago attu	9.42

* Calculated from the % denaturation in 10 minutes.

TABLE V. ALKALI DENATURATION OF HAEMOGLOBINS OF TREMATODES AND THEIR HOSTS.

Species	TIME (minutes)				
	1	4	8	12	16
Trematodes:					
Gastrothylax crumenifer	94.0	86.0	76.8	72.2	65.8
Gigantocotyle explanatum	96.0	90.0	83.7	80.0	76.0
Calicophoron calicophorum	95.7	85.7	74.3	64.3	55.7
Host:					
Bubalus bubalis	97.0	89.5	76.0	68.6	57.0
Trematodes:					
Fasciolopsis buski	-	-	-	90.0	80.0
Gastrodiscoides hominis	94.0	84.0	80.0	76.0	72.0
Host:					
Sus scrofa	88.4	70.5	55.8	44.2	33.7
Trematode:					
Isoparorchis hypselobagri	90.0	62.0	42.0	26.0	16.0
Host:					
Wallago attu	58.3	20.8	8.3	3.3	-

Values are percent undenatured haemoglobin remaining in the solution.

DISC GEL ELECTROPHORESIS:

The carbonmonoxy derivatives of trematodes and host haemoglobins were subjected to disc gel electrophoresis. The results are shown in Figs. 10-12, and for the sake of convenience they have been described below separately.

Cattle trematodes:

Upon electrophoresis, the cattle carbonmonoxyhaemoglobin appears as a single dense band and the Rm value for the same comes to 0.467.

The haemoglobins of Gastrothylax crumenifer and Cotylophoron cotylophorum constantly appeared as two separate bands, a more rapidly migrating one, which has been designated as Hb_1 , and the slower migrating one as Hb_2 thus suggesting the occurrence of more than one fraction of haemoglobins. The Rm values for haemoglobins of Gastrothylax crumenifer and Cotylophoron cotylophorum are 0.400 and 0.271 respectively, for the two fractions in each case.

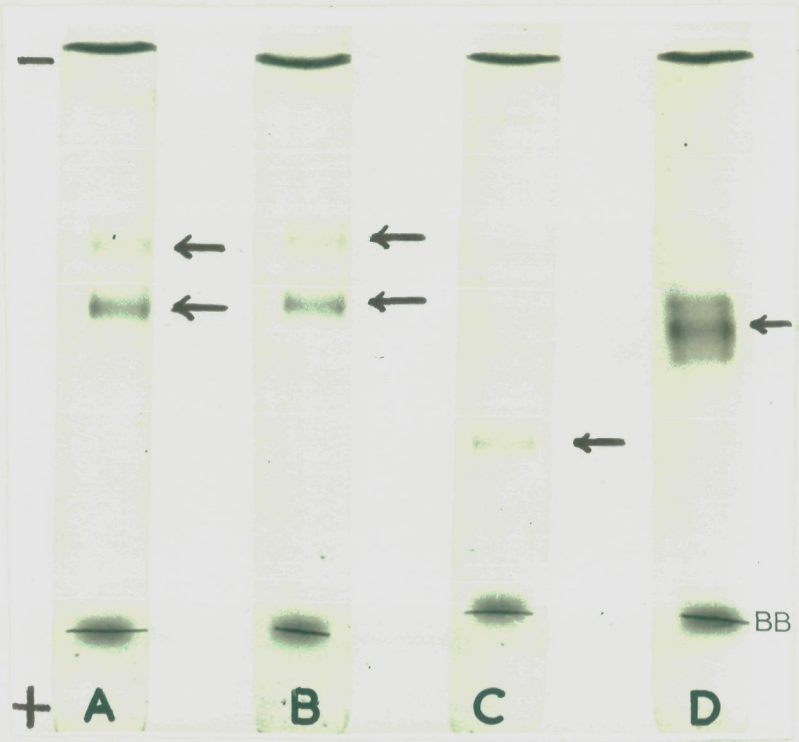

Fig. 10. Polyacrylamide gel electrophoretogram of carbonmonoxyhaemoglobins of cattle and cattle trematodes.

A. Gastrothylax crumenifer

B. Cotylophoron cotylophorum

C. Gigantocotyle explanatum

D. Bubalus bubalis

Arrows indicate haemoglobin bands (upper, Hb_2; lower, Hb_1). Origin (cathode) at top. BB = Bromophenol Blue.

However, the haemoglobin of Gigantocotyle explanatum revealed only a fast migrating band, the Rm value of this band is 0.661.

Pig trematodes:

The haemoglobin of pig on electrophoresis usually showed a single band, but however, sometimes an additional band was also observed. The Rm of the host haemoglobin is 0.430.

The haemoglobins of Fasciolopsis buski constantly appeared as a single band, whereas the haemoglobin of Gastrodiscoides hominis appears as two bands; the more rapidly migrating one is named as Hb_1 and the slower migrating one as Hb_2. The Rm value of Fasciolopsis buski haemoglobin comes to 0.65, while the Rm value of Hb_1 and Hb_2 Gastrodiscoides hominis haemoglobin fractions come to 0.60 and 0.40 respectively.

Fish trematode:

Fish carbonmonoxyhaemoglobin, on electrophoresis appeared as a single dense band and the Rm value of this band is 0.372.

Electrophoresis of Isoparorchis hypselobagri haemoglobin usually appeared as two bands but sometimes benzidine staining revealed 4 more bands in addition to the major one. Whether the additional bands are staining artefacts or suggests more than two fractions of a haemoglobin is difficult to ascertain at this

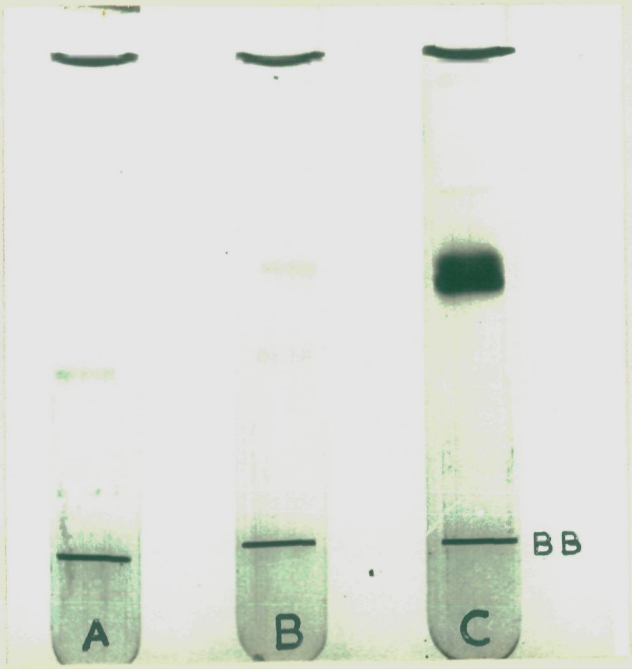

Fig. 11. Polyacrylamide gel electrophoretogram of carbonmonoxyhaemoglobins of pig and pig trematodes.

A. Fasciolopsis buski

B. Gastrodiscoides hominis

C. Sus scrofa

Arrows indicate haemoglobin bands (upper, Hb_2; lower, Hb_1). Origin (cathode) at top. BB = Bromophenol Blue.

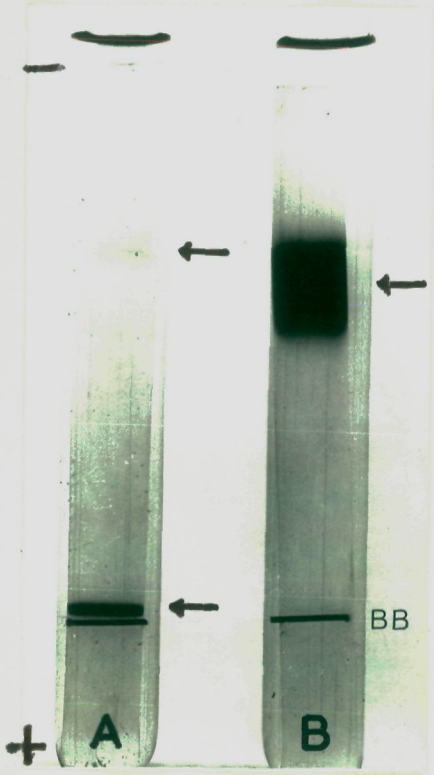

Fig. 12. Polyacrylamide gel electrophoretogram of carbonmonoxyhaemoglobins of fish and fish trematodes.

A. Isoparorchis hypselobagri

B. Wallago attu

Arrows indicate haemoglobin bands (upper, Hb_2; lower, Hb_1). Origin (cathode) at top. BB = Bromophenol Blue.

stage, and will be considered in further studies. The Rm values of the two major fractions of carbonmonoxyhaemoglobins of Isoparerchis hypselobagri named as Hb_1 and Hb_2 come to 0.972, and 0.342 respectively.

ELUTION PATTERN AND MOLECULAR WEIGHT ESTIMATION:

The column chromatography was employed mainly for two purposes, firstly to obtain an elution profile of the semi-purified haemoglobins of trematodes, and secondly, to determine the molecular weight of these proteins with the help of a calibration curve obtained by gel filtration of marker proteins of known molecular weights as shown in Fig. 13.

(1) Elution pattern:

The elution patterns of the oxyhaemoglobins of six different species of trematodes are shown in Figs. 14-19. It can be seen that different species of trematodes show different elution patterns and 2-6 peaks were obtained.

(a) Cattle trematodes:

The gel chromatogram of haemoglobins of *Gastrothylax crumenifer*, *Calicophoron calicophorum* and *Gigantocotyle explanatum* do not show identical elution profile indicating differences in the basic nature of the proteins. *Calicophoron calicophorum*

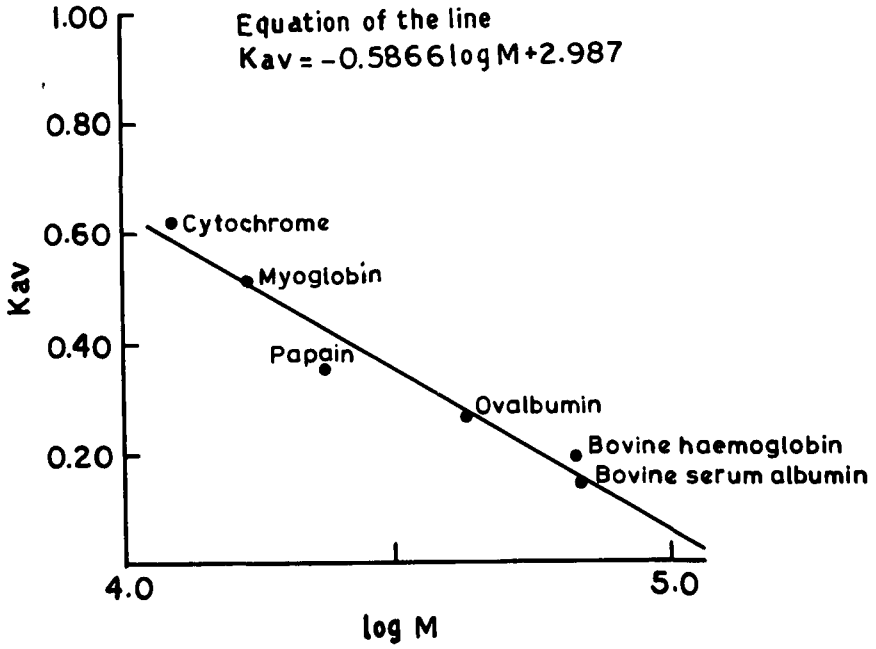

Fig. 13. Calibration curve for the determination of the molecular weight of trematode haemoglobins obtained by gel filtration on Sephadex G-100 column with proteins of known molecular weights.

oxyhaemoglobin is unique in showing six peaks with absorbances at 412 nm and 280 nm.

The major peak in all cases gives the characteristic absorption spectrum of the haemoglobin in the visible region with β , α and soret bands.

Gastrothylax crumenifer oxyhaemoglobin yields only two peaks, the major showing the characteristic absorption spectra in the visible region. In the case of Gigantocotyle explanatum three peaks were obtained, the major one showing all the characteristics of a true haemoglobin. 1*

(b) Pig trematodes:

Chromotography of both Fasciolopsis buski and Gastrodiscoides hominis oxyhaemoglobins revealed three peaks, two minor and a major peak, the latter gives the characteristic absorption spectra in the visible region for the haemoglobin pigment.

(c) Fish trematode:

As in the other cases, Isoparorchis hypselobagri oxyhaemoglobin yields two peaks, and on spectral examination in the visible region the major one revealed the characteristic absorption maxima in the β , α and the soret region.

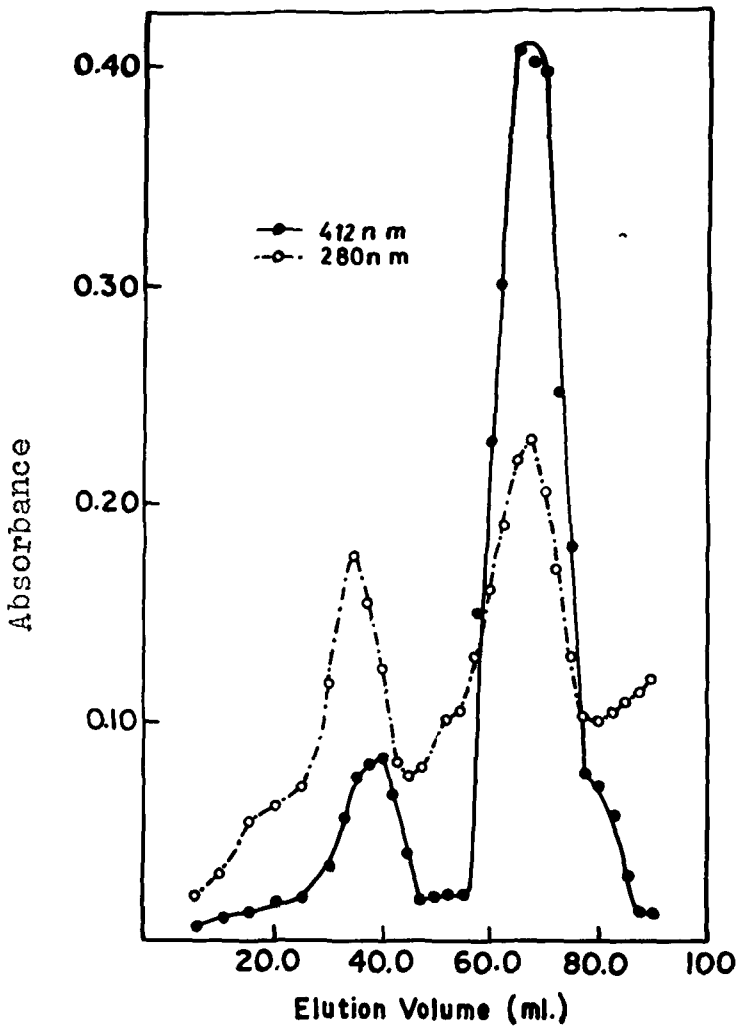

Fig. 14. Elution profile obtained by Sephadex G-100 gel filtration of Gastrothylax crumenfier oxyhaemoglobin ater partial punification by 55 to 75% ammonium sulphate saturation.

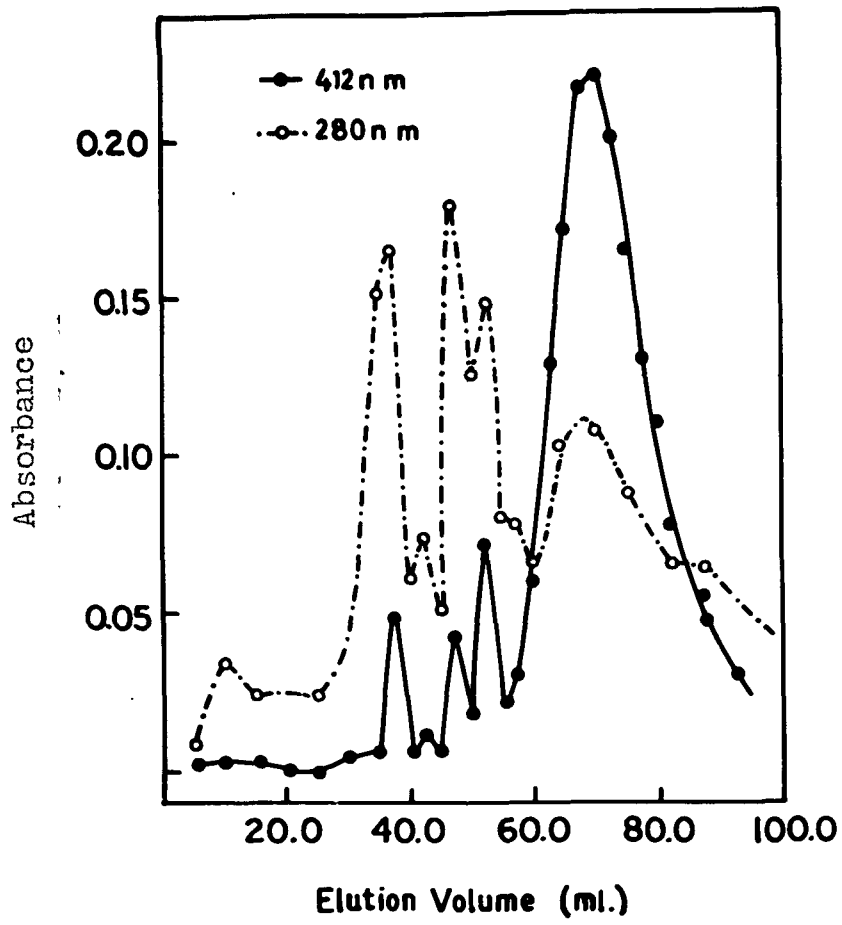

Fig. 15. Elution profile obtained by Sephadex G-100 gel filtration of Cotylophoron cotylophorum oxyhaemoglobin after partial purification by 55 to 75% ammonium sulphate saturation.

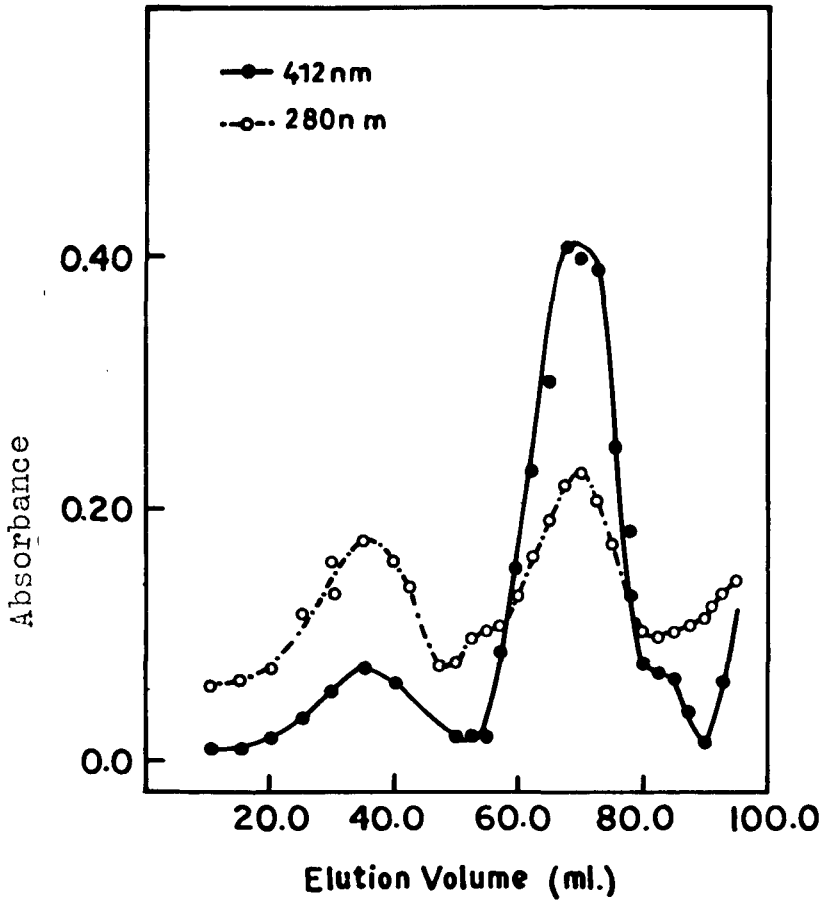

Fig. 16. Elution profile obtained by Sephadex G-100 gel filtration of Gigantocotyle explanatum oxyhaemoglobin after partial purification by 55 to 75% ammonium sulphate saturation.

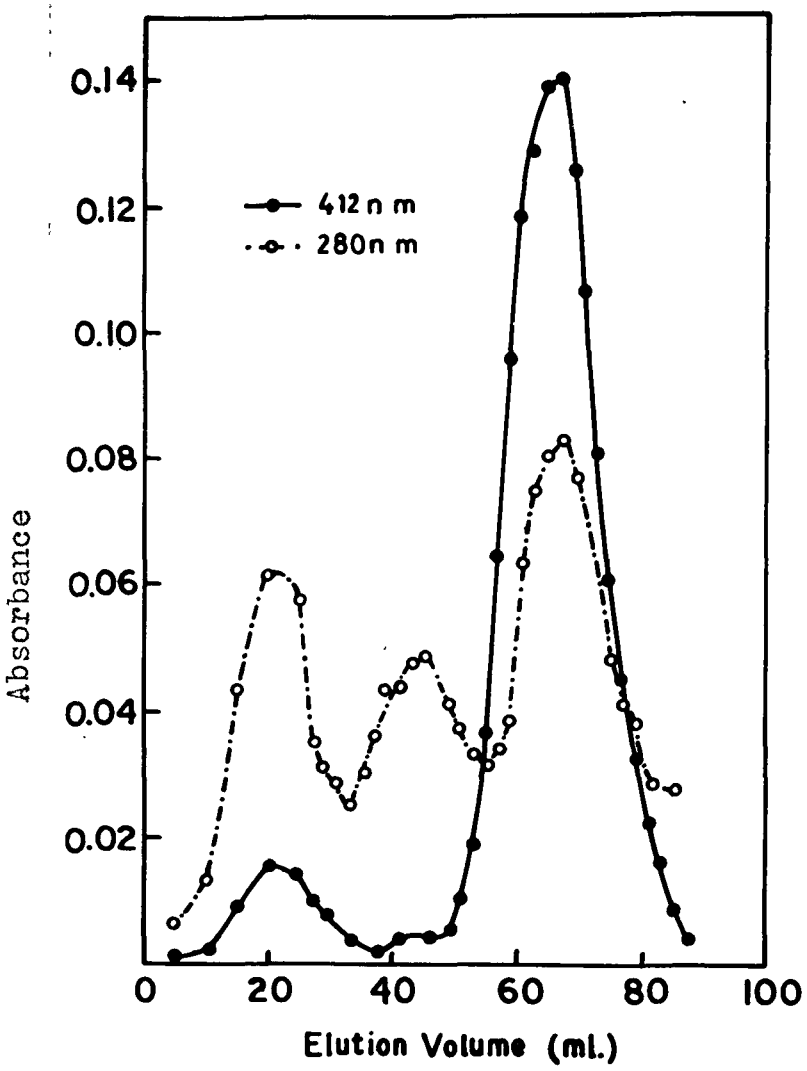

Fig. 17. Elution profile obtained by Sephadex G-100 gel filtration of Fasciolopsis buski oxyhaemoglobin after partial purification by 55 to 75% ammonium sulphate saturation.

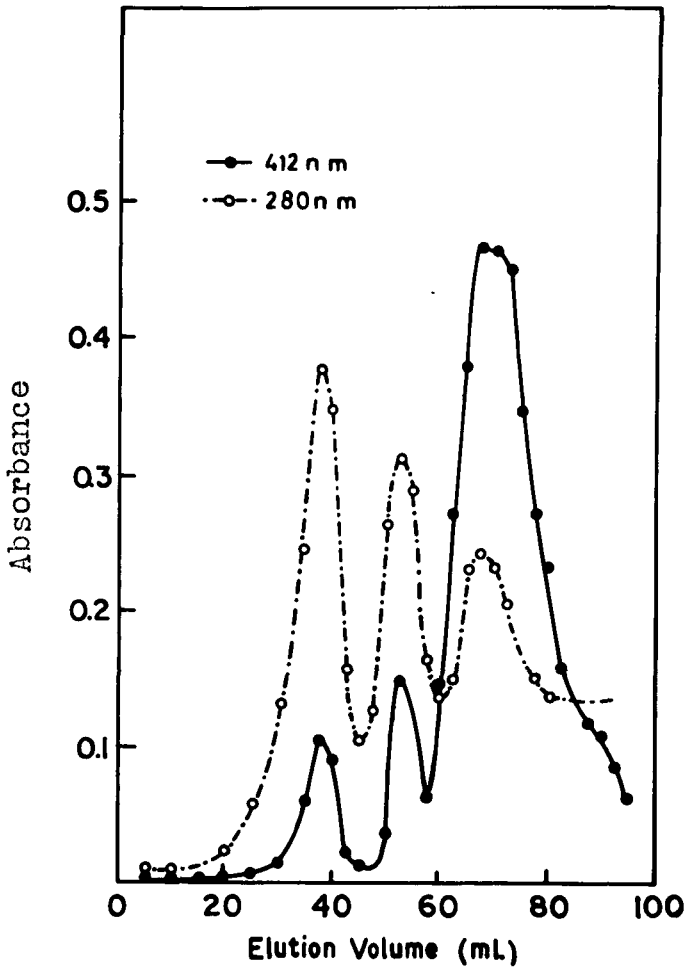

Fig. 18. Elution profile obtained by Sephadex G-100 gel filtration of Gastrodiscoides hominis oxyhaemoglobin after partial purification by 55 to 75% ammonium sulphate saturation.

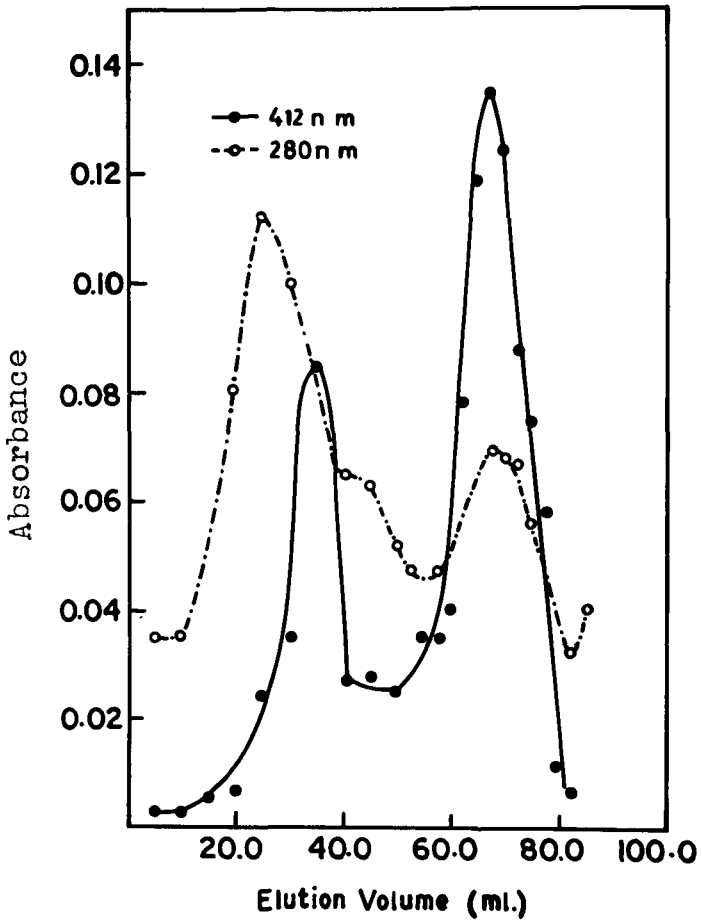

Fig. 19. Elution profile obtained by Sephadex G-100 gel filtration of Isoparorchis hypselobagri oxyhaemoglobin after partial purification by 55 to 75% ammonium sulphate saturation.

In all cases, the major peak obtained at 412 nm was considered to be trematode haemoglobin and was used for molecular weight estimations. The minor peaks are probably subunits of the major fraction. Such subunits are found due to disturbance of equilibrium in the gel column by diffusion of smaller subunit aggregates into internal cavities of the gel which are not accessible to larger aggregates. These subunits probably hook on to the contaminating proteins in a partially purified haemoglobin sample.

(2) Molecular weight:

As mentioned above the calibration curve was obtained by first eluting six marker proteins of known molecular weights on a Sephadex G-100 column. From their elution volumes, their K_{AV} values were calculated and were plotted against their known molecular weights on a semilog graph paper. A straight line relationship was obtained. The molecular weights of trematode oxyhaemoglobins were determined by calculating the K_{AV} values from the elution volumes of those fractions which showed the highest or maximum absorption at 412 nm. The K_{AV} values were extrapolated on the calibration curve to determine the molecular weights. The molecular weights of trematode oxyhaemoglobins range from 17,000 to 21,200 and the results are shown in Table VI.

TABLE VI. MOLECULAR WEIGHTS OF HAEMOGLOBINS OF TREMATODES AND THEIR HOSTS.

Species	Molecular weight
Trematodes:	
Gastrothylax crumenifer	17,000
Cotylophoron cotylophorum	21,200
Gigantocotyle explanatum	17,000
Host:	
Bubalus bubalis	68,000
Trematodes:	
Fasciolopsis buski	17,200
Gastrodiscoides hominis	17,000
Host:	
Sus scrofa	68,000
Trematode:	
Isoparorchis hypselobagri	17,000
Host:	
Wallago attu	68,000

DISCUSSION

SPECTROPHOTOMETRIC ANALYSIS:

The absorption of haemoglobin in the visible region of the spectrum depends essentially on the nature of the prosthetic group, i.e., porphyrin, which is a uniform part of the haemoglobin molecule in different animals. It is the protein moiety which differs in haemoglobins of different organisms. In the visible range, three peaks, γ, β and α are obtained at 410 nm, 540 nm and 580 nm respectively. The minor changes in absorption spectra depend upon factors such as hydrogen ion concentration, ionic strength, temperature and storage, and peak shifts of 1-2 nm are within experimental errors and should be ignored.

The absorption spectra of the porphyrins are so characteristic that in many cases they can be used for identifying and differentiating their kinds. This is especially true for haemoglobin and its derivatives which can be classified as ferrous ionic or ferric covalent compounds, according to the nature of their absorption spectra. Individual derivatives of haemoglobin, such as oxyhaemoglobin and carbonmonoxyhaemoglobin can be recognised by the exact absorption characteristics, with a

typical spectral shift (Lemberg & Leggi, 1969; Saunders et al., 1964). The derivative which is normally present in living tissues is oxyhaemoglobin which shows two absorption bands in the visible region, with peak maxima at 540 nm and 580 nm, and deoxygenated haemoglobin which has a peak maximum at about 555 nm. Although some other derivatives have somewhat similar spectra, none of these is likely to occur naturally, and demonstration of the conversion of oxyhaemoglobin to other derivatives makes identification of the haemoglobin certain. It is usual to show that oxyhaemoglobin, carbonmonoxyhaemoglobin and deoxygenated haemoglobin are formed in the ferrous state; and acid cyan-(cyanide) methaemoglobin in the ferric state. Reduced haemoglobin is ideally produced by deoxygenation in vacuo. Most haemoglobins have a greater affinity for carbon monoxide than for oxygen so that the carbonmonoxide derivatives form readily.

Physiologically active haemoglobins according to the classical definition are those involved in reversible oxygenation, namely oxyhaemoglobin and deoxyhaemoglobin. Many workers have used the absorption maxima values to distinguish between haemoglobins from different species. According to Lutz & Siddiqi (1967) "a careful and precise measurement of the spectra of trematode pigment should therefore help in its identification and a comparative study with the host haemoglobin may throw some light on the degree of their differences".

However, from the present work based on the detailed spectrophotometric analysis of haemoglobins and their derivatives from six different trematodes and their three different hosts, it is evident that absorption maxima cannot be used to distinguish between haemoglobins of different species of trematodes or between haemoglobins of parasites and hosts, since most maxima are obtained between 560 and 580 nm. However, spectrophotometric analysis can be of immense value in detecting haemoglobin in trematodes, even if present only in very small quantities. The haeme of trematode haemoglobin is identified as Fe protoporphyrin IX by the spectra of the reduced, alkaline, cyanide, and pyridine haemochromogens (Vernon & Kamen, 1959). The wavelength maxima of these derivatives are indistinguishable from those of the authentic protoporphyrin IX, and are different from those of cytochrome C haeme, the only compound with which they might easily be confused. The data for the spectral absorption of parasite haemoglobins in the visible region are shown in Table I to III. The results show the similarities and variations in the location of the absorption maxima. Nevertheless, the finding of virtually identical spectral maxima of parasite and host haemoglobins cannot be taken as proof that these haemoglobins are identical. In such a case it would be necessary to demonstrate that the haemoglobins are also identical in regard to other physico-chemical properties such as iso-electric point, molecular weight, alkali denaturation, electrophoretic mobility, amino acid sequence etc., etc.

The spectral study of haemoglobin in trematodes have been made by many workers such as Wharton (1938, 1941); van Grembergen (1948); Goil (1959, 1961); Freeman, (1963); Todd & Ross (1966); Lutz & Siddiqi (1967) and Cain (1969). The results of these workers and those of the present study show that the pigment under consideration is indeed haemoglobin. The peak absorption of the α, β and the soret bands occur at 574, 540, and 412 nm, and in all the six species of trematodes oxyhaemoglobin maxima correspond very closely with the known absorption values of oxyhaemoglobin of other invertebrates and vertebrates (Read, 1962; Wittenberg et al., 1965; Harper, 1967; Prosser & Brown, 1962; Cain, 1969).

The span between the bands of oxy- and carbonmonoxy derivatives for Gastrothylax crumenifer, Cotylophoron cotylophorum and Gigantocotyle explanatum is 40 A° and 60 A° respectively while that for host haemoglobin it is 60 A°; the haemoglobins of Fasciolopsis buski, Gastrodiscoides hominis have a span of 40 A°, and 60 A° respectively, which is comparable to the span of vertebrate haemoglobin (about 60 A°). Similarly the span of both Isoparamphis hyperboreri and Wallaga attu haemoglobins is 60 A°. The span difference was once thought to be of fundamental significance, but is now generally quoted merely as a further means of distinguishing various haemoglobins (Smith & Lee, 1965).

The absorption values for Gastrothylax armamifer and Fasciolopsis buski oxyhaemoglobins reported earlier (Goil, 1969; Cain, 1969) are quite different from those obtained in this study. These differences might be due to various factors; nevertheless, the values identify parasite pigment quite firmly as a haemoglobin. The values quoted by Wharton (1941) for Alloacanthoma magnum and Telorchis robusta need to be revised as they do not correspond with his Fig. (2) which gives an alpha band at 570 nm, and the beta band at 537 nm. The absorption values for Prosthogonus subtenuis (Freeman, 1963) is 570 nm and 543 nm for α, and β bands, which are very much similar to the values observed by van Grembergen (1949) for Fasciola hepatica and by Lutz and Siddiqi (1967) for Fasciola gigantica.

However, cyanmethaemoglobin provided with absorption maxima quite different from those obtained for host haemoglobins, or as reported in the literature. Usually this derivative in vertebrates shows a single broad band with a peak absorption at 538 - 540 nm. In all species studied, the trematode cyanmethaemoglobin showed two absorption maxima, both in the usual β and the α regions instead of a usual single absorption maximum at 540 nm. Similarly, Wittenberg et al. (1965) reported 2 absorption peaks at 570 and 540 nm in the case of Ascaris perienteric fluid cyanmethaemoglobin. The only plausible explanation to this seems that in ferrohaemoglobin, the haeme complexes possess a net

charge of one, which holds a negative group such as hydroxyl. In passing from ferrohaemoglobin to ferrihaemoglobin state the molecule looses its capacity to combine with O_2, CO and other molecules. In the present case it is possible that the change over from Ferrous iron (Fe^{++}) to the Ferric state (Fe^{+++}) is either incomplete or it does not occur at all.

Usually CN attaches to haeme at position 6 and inhibits binding with O_2. However, if haemoglobin conversion to cyanmet state does not take place, as appears to be the case with trematode haemoglobin, the latter is capable of binding with O_2 and thus storing it for use by the worms. Secondly, potassium cyanide, the major respiratory inhibitor, inhibits the activity of only cytochrome a_3, and consequently, it does not completely inhibit cellular respiration. This can be explained in two ways: (1) either the residual respiratory activity is due to cytochrome b (which is insensitive to carbonmonoxide and cyanide and the activity of which is independent of cyanide sensitive cytochrome a_3); (2) or, this residual respiratory activity is due to aerobic dehydrogenases which can transport hydrogen to oxygen without passing along the cytochrome system. These reactions involve flavoproteins, which have riboflavin as a prosthetic group. These flavoproteins are colourless in the reduced form and yellow in the oxidised form. They are widely distributed in animal tissues and play an important role in the hydrogen electron transport system.

Lazarus' (1960) found that cyanide stimulated rather than depressed the rate of respiration in Paramphistomum sevvi. It is obvious that the oxygen consumption can be inhibited in various ways. Firstly, the actual respiratory enzyme may be impaired when the cytochrome system is blocked by cyanide. Secondly, the overall oxygen consumption can be cut down to various degrees depending upon the availability of alternate pathways, if a compound inhibits a certain metabolic sequence above the stage where hydrogen is activated. Therefore, the increase in the rate of oxygen utilization in Paramphistomum sevvi as observed by Lazarus' (1960) may be due to the presence of a functional cytochrome system or otherwise the residual activity may be due to aerobic dehydrogenases which can transport O_2 without passing along the cytochrome system.

Contrary to Lazarus' (1960) observation, cyanide inhibition of O_2 consumption has been observed in the case of three species of trematodes: Gigantocotyle explanatum, Gastrodiscoides hominis and Cotylophoron cotylophorum in this laboratory (unpublished results). This has also been shown in the case of Schistosoma mansoni by Bueding (1950), Fasciola hepatica by van Grembergen (1949) and Dicrocoelium dendriticum by Eckert and Lehner (1971). It is therefore suggested that one should not accept Lazarus' (1960) findings without further reinvestigation.

It appears from the result of the present investigation that trematode pigments are resistant to cyanide conversion, since

two peaks in the usual β, α range are obtained in the case of all the six species of trematodes compared with the single peak in the case of their vertebrate hosts haemoglobin.

It may be of interest to mention here that the author tried to convert trematode and their host haemoglobins into methaemoglobin by the addition of potassium ferricyanide, which usually give a single absorption peak in 630 - 640 mμ region, such a single peak was obtained in the case of vertebrate host haemoglobins but not in the case of trematode haemoglobins. This means that the trematode haemoglobins are resistant to conversion into methaemoglobin. In this way one can conclude with certainty that the trematode haemoglobins are distinctly different from the host haemoglobins as far as their conversion to cyanmet and methaemoglobin is concerned.

ALKALI DENATURATION:

The alkali denaturation of oxyhaemoglobins of trematodes has never been studied before, though this physico-chemical property of haemoglobin has been studied in other animals. It is a good indication of the presence and absence of alkali labile components in a particular haeme protein. Both the degree and rate of denaturation can be utilised to indicate differences between haeme protein molecules of similar nature. Not only

various species show interspecific differences in the kinetics of alkali denaturation of their haemoglobin molecule but different stages of the same species show heterogenity and homogenity of the haemoglobin molecule. Alkali denaturation of larval and adult haemoglobins of rainbow trout revealed that the larval haemoglobins are found to contain more alkali labile components than the adult ones (Iuchi, 1073).

The differences in susceptibility to alkali denaturation most probably depend on the number, ionization and hydration of buried side chains at a particular pH. The latter plays an important part in the denaturation of the haemoglobin molecule. The information about the half time of alkali denaturation of a protein is an indication of the differences in the amino acid sequence of the side chain. According to Perutz (1974) the rate of alkali denaturation depends upon the presence of certain amino acids, e.g., at pH 12.7 and 20°C the half times of the denaturation reaction of canine and adult human cynomet haemoglobins are similar, 20 to 25 s as compared to 11 s. This is consistent with the presence in both species of Cys 0llα, Cys 14 β and Tyr# 8 β (Jones et al., 1972). In a similar manner the haemoglobin of the various species of monkeys contains little or no alkali resistant haemoglobin and Cys 0llα, Cys 14 β and Tyr#8 β are all present. The presence of Cys 0llα, Cys 14 β and Tyr 8 H β render haemoglobin of different species susceptible to alkali.

In the present study the denaturation of haemoglobins of six different species of trematodes studied under similar conditions of temperature and pH revealed that all the six species of trematode haemoglobins differed not only from host haemoglobins but from each other as far as susceptibility to alkali denaturation was concerned. If one compares the extent of the denaturation of trematode haemoglobins by alkali, it is obvious that, Isoparorchis hypselobagri is most alkali labile followed by Gastrothylax crumenifer, Gigantocotyle explanatum, Gastrodiscoides hominis and Fasciolopsis buski (see Table V). In 16 minutes nearly 84% of Isoparorchis hypselobagri haemoglobin is denatured whereas in the same period of time only 20% denaturation takes place in the case of Fasciolopsis buski haemoglobin.

Similarly the three host haemoglobins also differ from each other in the extent and rate of alkali denaturation. Fish haemoglobin is most susceptible and is completely denatured whereas cattle haemoglobin is least susceptible. However, in all six species of trematodes, the denaturation of oxyhaemoglobin follows the first order kinetics, which is indicative of the homogenity of the haemoglobin molecule under discussion. The variation in the rate of alkali denaturation can be explained on the basis of the variations in the amino acid sequences of a particular haeme protein. The present experiments therefore show that the various trematode haemoglobins are distinctly different from host haemoglobins as well from each other.

DISC ELECTROPHORESIS:

The electrophoretic mobility of proteins is a convenient and important characteristic and can be used to study their heterogenity. Haemoglobin from an animal can be studied not only for its physico-chemical properties but also for the study of its components.

Proteins are composed of chains of approximately twenty different kinds of amino acids linked together in a definite sequence. The sequence of the amino acids in a protein chain is determined by genes. Haemoglobin, serum albumin, and ovalbumin are proteins with definite structure that vary from species to species. The differences between species are, for many proteins proportional to the genetic differences between the species that have arisen during evolutionary divergence. Thus, two closely related species tend to have similar or partially similar proteins, while the proteins of distantly related species are less similar. By comparing the properties of the same type of proteins from different species it is possible to obtain information about their evolutionary significance.

The electrophoretic studies on trematode haemoglobins have been made in isolated one or two cases and our knowledge in this field of study is almost fragmentary. Lutz & Siddiqi

(1967) showed for the first time by using paper electrophoresis as well as spectrophotometry, that the haemoglobin of Fasciola gigantica is a true porphyrin pigment which is distinct from the host haemoglobin. More recently Cain (1969a,b,c) using more reliable and modern techniques reached to the same conclusion. However, Cain (1969) claimed to have reported for the first time that haemoglobin of Fasciolopsis buski, possesses two fractions and wrongly reports that only one haemoglobin fraction was reported in the case of Fasciola gigantica by Lutz and Siddiqi (1967). The latter workers are certainly the first authors who showed that Fasciola gigantica possesses two fractions as can be seen in their Fig. 3.

From the results presented earlier it can be concluded that electrophoretic mobilities of haemoglobins of all the three species of cattle trematodes, Gastrothylax crumenifer, Gigantocotyle explanatum and Cotylophoron cotylophorum differ from the electrophoretic mobility of haemoglobin of their host, Bubalus bubalis. In this host parasite system, host haemoglobin appears as a single slow migrating band, and remains nearer to the cathodal end of the gel, similarly the haemoglobin of rumen dwelling trematodes, i.e., Gastrothylax crumenifer and Cotylophoron cotylophorum also appear nearer the cathodal end, however, haemoglobin from each species appears in two bands rather than one. The relative mobility of both these fractions i.e., Hb_1 and Hb_2 is less than the relative mobility of the host

haemoglobin which indicates that the trematode haemoglobins have low isoelectric point than the host haemoglobin, and possess large number of basic amino acids. However, the Gigantocotyle explanatum haemoglobin, on electrophoresis, appears as a single fast migrating band whose Rm value is more than the host haemoglobin, thus suggesting that it contains more of the acidic amino acids. The differences in electrophoretic mobilities may be explained either due to the differences in amino acid composition or sequence or due to the specific folding of the polypeptide chain. The former possibility appears to be the case.

In the second host parasite system i.e., pig and its trematodes, the host haemoglobin migrates slowly and appears as a single band, while the Gastrodiscoides hominis haemoglobin appears to be composed of two fractions, the relative mobility of both these fractions is more than the relative mobility of the host haemoglobin. Similarly the migration of Fasciolopsis buski haemoglobin is even slightly more than the relative mobility of the Hb_1 fraction of the Gastrodiscoides hominis haemoglobin, thus suggesting that both the fractions of Gastrodiscoides hominis haemoglobin as well as the haemoglobin of Fasciolopsis buski are rich in amino acids i.e., the trematode pigment is lower in leucine, lysine and histidine and higher in aspartic acid (Cain, 1969).

In the third host parasite system, i.e., the fish and its trematode, the host haemoglobin appears as a single slow moving dense fraction and it stays close to the cathodal end. The haemoglobin of Isoparorchis hypselobagri appears as two fractions, one major and one minor. The major band which is called Hb_1 fraction is very fast moving and migrates immediately following the band of the bromophenol blue tracking dye. The second fraction called Hb_2 of the Isoparorchis hypselobagri haemoglobin migrates at a much slower speed, the Rm of the Hb_2 fraction of the Isoparorchis hypselobagri haemoglobin is less than the relative mobility of the host haemoglobin, thus indicating the occurrence of two types of haemoglobins, one being basic in nature, and the other acidic.

From the foregoing account, it can be seen that the host haemoglobins give a slow migrating band in all the three cases under study, while the trematode haemoglobins except Gigantocotyle explanatum and Fasciolopsis buski haemoglobin seem to consist of two fractions, a fact reported in the case of Fasciola gigantica [HI also by Lutz & Siddiqi (1967).

It appears that the trematode haemoglobin consists of at least two fractions and wherever only one fraction has been reported, the occurrence of a second band must be reinvestigated. Cain (1969) also reported the appearance of an additional band in the case of Fasciolopsis buski but was not sure to call it a second fraction. He considered it an artefact of staining. While

this may be true in the case of Isoparorchis hypselobagri also where four additional but faint bands were once obtained by the present author. The existence and visibility of minor bands also depend on the concentration of haemoglobin sample subjected to electrophoresis. In future studies this point must be kept in mind. An interesting feature of the present study is that out of the six trematode parasites four are amphistomes, living in three different habitats in two different hosts, and in all cases except Gigantocotyle explanatum, the amphistome haemoglobin appears as two fractions revealing a remarkable parallelism between the electrophoretic patterns of closely related species. This feature of the present study indicates that Cotylophoron cotylophorum, Gastrothylax crumenifer and Gastrodiscoides hominis are closely related to each other than to Gigantocotyle explanatum in spite of the fact that the latter species lives in the same host as the first two species in the former group. In other words the biochemical similarities support taxonomic relationships. Isoparorchis hypselobagri haemoglobin is distinctly different from the other five trematode haemoglobins as far as electrophoretic mobility is concerned and if one looks at the taxonomic relationship, it will become obvious that Isoparorchis hypselobagri under the family Isoparorchidae is closer to the Hemiuridae than to Paramphistomatidae or Fasciolidae.

ELUTION PATTERN AND MOLECULAR WEIGHTS OF TREMATODE HAEMOGLOBINS:

The chromotographic profile of the elution pattern of the trematode haemoglobin both in the ultraviolet and the soret region showed that two to four minor contaminants are present, for which minor peaks were obtained. They were eluted with higher molecular weight non-haeme proteins in addition to the major haeme fraction.

The most fundamental property of any protein molecule is its molecular weight. The determination of molecular weights of haemoglobins from six different species of trematodes living in three different hosts, revealed that the molecular weights in all the six species ranged between 17,000 to 21,200. A value that is very similar to that observed by Cain (1969) for Fasciolopsis buski haemoglobin, the only trematode haemoglobin, whose molecular weight has so far been studied. Cain (1969) showed that the molecular weight of Fasciolopsis buski haemoglobin is 15,000 to 16,000 when determined by plotting Rf values obtained by SDS electrophoresis against known molecular weights of marker proteins, while the estimation of molecular weights by Sephadex G-100 revealed a molecular weight of 17,000, a value which corresponds very closely to known molecular weight of sperm whale myoglobin, thus suggesting that trematode haemoglobin is very much closely related to myoglobin. Cain (1969) further reported that Fasciolopsis buski protein resembles vertebrate

myoglobin in lacking subunits, having one haeme and containing a similar number of amino acids, but half cystine and taurine were detected in the preparation of trematode haemoglobin. There are evidences that indicate that haeme proteins of trematodes are pigments capable of reversibly binding oxygen. Hence the recommendation of Keilin & Hartee (1951) is followed here in terming them 'trematode haemoglobin'. Although the trematode haemoglobin has many of the properties of myoglobin, the etymology of that term makes it inappropriate for haemoglobins not restricted to muscle tissues. Resemblance between trematode haemoglobin and myoglobin suggests that the two may have similar functions, i.e., to provide oxygen reserve during periods of hypoxia. In the majority of all the cases it is believed that the role of trematode haemoglobin is to supply the atmospheric oxygen to the tissues; in addition to this function it is thought that oxygen storage may also be one of its functions. Since haemoglobin is an oxygen binding pigment, it either maintains a continuous supply of oxygen to the tissues or it provides an oxygen reserve. This is so in case of both circulating as well as tissue haemoglobins. The passage of oxygen through the tissue pigment at low oxygen tension has been shown by Scholander (1960). The tissues which are nearer to the surface areas and which have a higher oxygen concentrations become more oxygenated compared with those tissues which are below the surface and are least oxygenated. As a result, their exists an

oxygen gradient from high to low oxygen concentrations. In this way the oxygen molecule is passed down the chain when haemoglobin dissociates. The affinity of parasite haemoglobin to oxygen is so high that they are completely saturated at 1 to 2 mm pO_2 (Lee & Smith, 1965). This clearly indicates that as far as function of haemoglobin in trematodes is concerned it undoubtedly appears to play a role in oxygen transport as in vertebrates generally.

The relation between the haemoglobins and myoglobins is apparent not only in tertiary structure but also in primary structure - primitive haemoglobins (cyclostomes) still consists of a single peptide chain with a molecular weight of 17,000. Chemical examination of the haemoglobin of Lamprey fluviatlis has shown that its amino acid sequence is closer to that of myoglobin than to that of mammalian haemoglobins; in fact, this primitive haemoglobin yields some tryptic peptides which are very similar to those obtained from myoglobin.

Being a primitive metazoan group, and the lowest bilatria, the trematodes would seem well suited for investigating biochemical evolution of the haemoglobin molecule. The molecular weight of known haemoglobins range from 13,000 to 1×10^6.

According to Ingram (1963) the haemoglobin molecule was ordinarily much simpler and that the molecule initially consisted of a single peptide chain. The ancestral molecule was probably

similar to myoglobin and had an almost identical tertiary structure. In order to explain the appearance of new molecules; i.e., peptide chains, it was proposed that gene duplication took place, each gene corresponding to one of the peptide chains, which were originally identical. An important point in the theory is the proposal that from this time on the genetic material developed independently by undergoing different mutations. It is supposed that gene duplication took place several times leading to the existence of the present α, β, γ and δ chains. At the same time two physical factors must have played a very decisive role. They were the formation of the dimers and tetramers of the peptide chains. The second important physical factor was the appearance of haeme interaction, which brought with it a marked increase in the physiological effectiveness of the molecule. It is believed that haemoglobins must have appeared at some time in the course of their evolutionary development probably independently in some of the wide range of forms in which it is now found. It is possible that haemoglobin arose through radical modification of a pigment of the cytochrome type, though a more likely first event is perhaps a chance association of protohaemo with a globular protein of molecular weight of 17,000 to give a pigment in which haeme is linked to protein through the central iron atom, and is capable of combining reversibily with oxygen. Once such a pigment arose, it is likely to have been physiologically advantageous especially in primitive multicellular organisms and

therefore to have been retained in subsequent evolutionary development. In vertebrates, one type of haemoglobin would have remained in muscle as a storage pigment with a high oxygen affinity and with no Bohr effect and another evolved independently in red blood cells. Wald (1962) has emphasised that two developments have greatly increased the efficiency of haemoglobin as oxygen carrier. The first is the establishment of the Bohr effect, which facilitates liberation of oxygen into the tissues. This is the stage reached in lampreys today. The second is association of units having molecular weight of about 17,000, to give first aggregates of two units (possibly present to some extent in hagfish) and then of four units as are found in all higher vertebrates. The association permitted of haeme interaction and the development of the characteristic sigmoid oxygen equilibrium curve, which allows a nice adjustment of loading and unloading tensions to meet the need of organisms throughout the development of the haeme group and its attachment to globin have remained evidently unchanged as indicated by the very close similarity of the absorption spectra of trematodes, lampreys and mammalian haemoglobins. Adaptation has been achieved by progressive modification of the protein subunits through repeated mutations.

GENERAL CONCLUSIONS:

Since trematodes live as parasites inside their host, one may ask whether there is any relationship between the haemoglobins of the parasite and that of its host. Evidently, at present no worker has shown any direct connection between parasite and host haemoglobin. It could be genuine haemoglobin of the parasite (endogenous) or perhaps a form of modified host's haemoglobin (exogenous) or built up by the parasite from simple units derived from the host haemoglobin.

The manner of origin of haemoglobin as an oxygen carrying pigment is still a matter of conjecture. Since the higher animals synthesise haemoglobin from relatively simple substances such as amino acids and iron, it may be presumed that parasites carry out similar synthesis employing the metabolites generally present in the microenvironment and not by absorption of the preformed haemoglobin of host origin. However, the acquisition of host haemoglobin by a trematode is not unlikely, since ferritin, a larger molecule than vertebrate haemoglobin can readily enter Fasciola hepatica tegument (Bjerkmann and Thorsell, 1964). Trematodes may therefore be capable of absorbing host haemoglobin and altering its physico-chemical characteristics. This fact is further supported by the fact that one of the trematodes under study, Isoparorchis hypselobagri exclusively feeds on fish blood since no other nutrients are available in the swim bladder

of the host. Cain (1960) using labelled amino acids gave the first direct evidence that Fasciolopsis buski synthesises at least protein moiety of haemoglobin, but no direct evidence for haeme synthesis has yet been put forward. Since every cell of aerobic organisms including bacteria is capable of synthesising different hematin catalysts, such as the components of the cytochrome system and catalase, every cell appears to be a potential carrier of haemoglobin. The limiting factor in the distribution of this pigment in nature cannot therefore be due to the synthesis of the haeme nucleus but to the fact that not all haeme producing cells are capable of synthesising the highly specific proteins which, when combined with haeme, form compounds possessing the remarkable property of reversible oxygenation. All parasite haemoglobins studied so far in some detail differ from the haemoglobin of their hosts in one or several characteristics. The colour of the worm when maintained in vitro remains unchanged (e.g., Dastronemylides sp. larvae contain haemoglobin up to four years in vitro according to von Brand and Simpson, 1945). Further analysis showed that the worms depend for haemoglobin synthesis on the availability of metalloprophyrins containing at least one vinyl group among the side chains. Smith and Lee (1963) developed the idea that Ascaris can in its intestinal cells degrade hematin to bile pigments which would then be used as substrate for haemoglobin synthesis. However, Perez and Bloch-Raphael (1965) take the orthodox view that these pigments are derived

from host haemoglobin destruction rather than considering them as substrates for synthesis. It does not appear plausible that these pigments are byproducts of nutrition since the haemoglobin persists in parasites for sometime in vitro in haemoglobin free media, and moreover the host of Prosthogonus subtenuis contains no haemoglobin (Freeman, 1963) although the parasite does.

The precise nature of the food utilised by the trematodes is still controversial and most of the work has centred on Fasciola hepatica, and many workers believe that blood forms the dominant element of the diet (Weinland and von Brand, 1926; Stephenson, 1947; van Grembergen, 1950; Pearson, 1963). On the other hand, Muller (1923) concluded from his histological studies that Fasciola hepatica feeds on bile duct epithelium and its contents, and migratory host leucocytes. Dawes (1961a,b,c, 1962, 1963a,b,c) has supported this point of view and calls Fasciola hepatica a tissue feeder. Rogers (1940) reported that Schistosoma japonicum ingests blood and hematin is an end product of digestion.

However, in the opinion of the present author, it is not unlikely that the haemoglobin present in the parasite is its own and not derived from its hosts, as there are large number of invertebrates in which haemoglobin has been reported either as intravascular haemoglobin or tissue haemoglobin which is of endo-

genous origin. Therefore, a pertinent querry is: why cannot trematodes possess haemoglobin of endogenous origin? Just because they are parasites and live inside a host, whatever they may possess is considered to be of host origin. When trematodes can synthesise their own various proteins as any other organism and which are specific to them, so can they produce their own endogenous haemoglobin, and it appears that the trematode haemoglobin acts more as a myoglobin having storage and facilitated diffusion functions. However, detailed knowledge of the exact function of trematode haemoglobin will depend upon its oxygen affinity, localisation and the oxygen tension of the environment. Although the exact picture could emerge only after the amino acid analysis of the trematode haemoglobin, and in most cases this is not known except in Fasciolopsis buski. The composition of which resembles the sperm whale myoglobin with respect to several amino acids, although the same number of residues in the two is a coincidence.

Recently Rama and Tuchschmid (1974) isolated and characterised a haeme-containing protein from Diarrenalium dendriticum by employing chromatography on Sephadex G-100 and DEAE-Sephadex. These workers demonstrated by NH_2-terminal and COOH-terminal sequence analysis and amino acid composition that the Diarrenalium haeme protein is neither the host haemoglobin nor myoglobin. In other words this work also supports the fact that in this case also the trematode pigment is parasite's own endogenous haeme protein.

As far as the nature of the trematode haeme protein is concerned, one has to examine the differences between the myoglobin and haemoglobin molecules. The myoglobin differs from the haemoglobin in the following manner:

Myoglobin is found in tissues whereas haemoglobin is found in cells; myoglobin has a molecular weight of 17,000 and haemoglobin has a molecular weight of 68,000 or more; myoglobin functions in the storage of oxygen whereas haemoglobin is used to transport oxygen; myoglobin becomes oxygen saturated at very low partial pressures of oxygen, whereas haemoglobin molecules become saturated at higher pO_2. The dissociation curve is a hyperbola in the case of myoglobin and sigmoid in case of haemoglobin. When one keeps in mind the above facts, it becomes apparent that the trematode haeme pigment deserves to be called a myoglobin rather than a haemoglobin. In future it is proposed that the trematode protoporphyrin IX containing proteins should be designated as trematode myoglobin.

SUMMARY

1. The chemical, physicochemical as well as physiological properties of haemoglobins isolated from six different species of trematodes from three different hosts were investigated and compared with their host haemoglobins.

2. The spectral characteristics of haemoglobins indicate that both the trematodes and the host haemoglobins contain porphyrin IX as the common prosthetic group and proves beyond doubt that the trematode pigments are porphyrin proteins.

3. The haemoglobins of all the six species of trematodes and their hosts under study gave similar absorption peak maxima. Distinct differences were however seen in the nature of the spectral curves of cyanmet haemochromogens and in all cases the trematode pigment derivatives give two absorption maxima in the β , and the α region, whereas in the case of host haemoglobin, only a single broad peak in the 536-540 region was obtained.

4. The homogenity of trematode as well as of host haemoglobins seems to be reflected in the denaturation kinetics of oxyhaemoglobins in an alkaline solution at pH 12.0. The alkali

denaturation of both the trematode and the host haemoglobins follow the first order kinetics.

5. Polyacrylamide disc gel electrophoresis revealed two haemoglobin fractions in Gastrothylax crumenifer, Cotylophoron cotylophorum, Gastrodiscoides hominis and Isoparorchis hypselobagri and only one fraction in the case of Fasciolopsis buski and Gigantocotyle explanatum. In general, the appearance and properties of the haemoglobin components correspond to the taxonomic relationship of the species.

6. The trematode haemoglobins were chromotographed and their molecular weights were determined on a calibrated Sephadex G-100 column. The molecular weight of the trematode pigments range from 17,000 to 21,200 compared with 68,000 in the case of their vertebrate host haemoglobins.

7. It can be concluded that the trematode haemoglobin exists in a monomeric form, with a single haeme group and a free sulphydryl group in each molecule.

8. The origin and evolution of the haemoglobin molecule is discussed. The first haemoglobin is believed to have been a protein of molecular weight of about 17,000. This was a precursor of myoglobin, a pigment mainly concerned with oxygen storage in muscle and haemoglobins of all vertebrates. Two main events characterized the evolution of the latter. The first was the

acquisition of a Bohr effect. Such a haemoglobin is found in lampreys even today. The second was the structural change which allowed aggregation of two pairs of unlike haemoglobin molecules into a unit of molecular weight of about 66,000 to 68,000.

9. From the results of the present investigations it appears that the trematode haeme pigment is of endogenous origin synthesized by the parasite itself and is a myoglobin rather than a haemoglobin.

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